

**STUDIES ON THE INVASIVE BEHAVIOR
OF MAMMALIAN CELLS *IN VITRO***

INAUGURAL-DISSERTATION

zur

Erlangung der philosophischen Doktorwürde

vorgelegt der

PHILOSOPHISCHEN FAKULTÄT II

der

UNIVERSITÄT ZÜRICH

von

CHRISTIAN SCHMIDHAUSER

von Hessenreuti (TG)

Begutachtet von

Prof. Dr. R. W. Parish

Prof. Dr. G. A. Dunn (London)

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INTRODUCTION

Metastatic tumor cells are characterized by quantitative alterations in cell surface and other properties that confer on them the ability to invade, implant, survive and grow in another organ. Metastasis is also determined by a variety of host factors that prevent or stimulate metastatic processes. The emergence of diversified cell subpopulations in malignant tumors ensures that some cells will become highly metastatic, resulting in tumor progression towards characteristics which are the most favorable for survival and growth. Unknown mechanisms may be altered by mutational or non-mutational modifications that individually influence cells within a malignant neoplasma.

Primary solid tumors are benign and it is the appearance of the metastatic phenotype that eventually kills the host. An understanding of the mechanisms leading to uncontrolled cell proliferation will presumably derive from the study of oncogenes. However, the biochemical changes critical for the development of metastatic potential are poorly understood.

Attempts to recognise mechanisms required for metastasis are generally based on the analysis of the sequence of events involved (Liotta et al., 1986). The metastatic cascade includes the release of cells from the primary tumor followed by their invasion of the basement membranes to reach the interstitial stroma. Subsequently, there is invasion of the basement membrane and endothelial layer of the blood vessel wall and dissemination via the blood or lymphatics. These cells are then arrested in capillaries of another organ by adherence to endothelial cells, luminal surfaces and / or exposed basement membranes. Finally, the cells again penetrate the vascular endothelium and / or its surrounding basal lamina and proliferate in the parenchyma of the organ.

In an attempt to identify molecular changes leading to the metastatic phenotype a variety of normal and invasive cells have been compared. A bewildering complexity of differences have been described, although, in most cases, convincing evidence for a role in metastasis has been lacking.

DNA transfection experiments are being used to search for specific genes whose activation or repression can lead to the metastatic phenotype. However, the results to date are contradictory (Bernstein and Weinberg, 1985; Greig et al., 1985; Bradley et al., 1986) and it appears that the transfection process itself can alter the metastatic properties of neoplastic cells (Kerbel et al., 1987).

Considerable attention has been focussed on the cell surface (Nicolson, 1984). It seems likely that changes in plasma membrane constituents and structure may be involved in the spread of tumor cells. *In vivo* studies using B16 mouse melanoma variants showed that plasma membrane vesicles from highly metastatic tumor cells can modify the metastatic

behavior of poorly metastatic sublines when fused with the latter (Poste and Nicolson, 1980). The mechanisms by which these changes bring about the altered behavior are not yet clear. The preferential metastasis of tumors to various organs in the body (Weiss, 1976; Fidler, 1978; Fidler and Hart, 1982) suggests cell surface differences on endothelial cells derived from different sources can be distinguished by tumor cells (Auerbach and Joseph, 1984). It follows that tumor cells express surface proteins capable of facilitating their adhesion to the vascular epithelium, the first step to crossing this barrier. The extravasation of lymphocytes depends on just this kind of specific binding to endothelial cells (Chin et al., 1983). Adhesion of tumor cells to subendothelial matrix is presumably also a prerequisite for extravasation (Kramer and Nicolson, 1979). Proteins that may be involved in such adhesion have been identified on mouse melanoma cells (Vollmers and Birchmeier, 1983). The forces keeping normal cells (or benign tumor cells) localized to appropriate tissues are not understood, but may involve cell adhesion molecules such as N-CAM (Greenberg et al., 1984).

Invasiveness of the extracellular matrix by tumor cells has been studied *in vivo* and *in vitro* (Jones and DeClerck, 1982) and correlations found between the production of hydrolytic enzymes such as proteases (Dano et al., 1985; Mignatti et al., 1986), collagenases (Liotta et al., 1982) and heparanases (Nicolson et al., 1985). However, this type of invasiveness accounts for only a part of metastatic behavior (Terranova et al., 1986).

Our work was focussed on contact inhibition of locomotion and cellular invasion. This type of invasion relates to the contact interaction between moving cells (Heaysman, 1978; Abercrombie, 1979; Paddock and Dunn, 1986). There is considerable evidence that the behavior of cell types during and following collision *in vitro* mimics aspects of certain fundamental biological processes, including malignant invasion (Abercrombie, 1979).

We chose to use Abercrombie's confronted explant technique to study the invasive behavior of cells *in vitro* (Abercrombie, 1975; Abercrombie and Heaysman, 1976; Steinemann et al., 1984ab). Recent work in this laboratory identified a 37kd protein which was involved in the invasive behavior *in vitro* of mouse fibrosarcoma cells (Steinemann et al., 1984ab; Parish et al., 1987). Monoclonal Fab directed against this antigen were found to inhibit the invasivity of these cells. The present work aimed to extend these results and had the following specific goals:

- (1) To screen various metastatic and non-metastatic cell lines for the presence of p37 and to determine whether there existed a consistent correlation between p37 and invasion *in vitro*.
- (2) To determine whether Fab specific for p37 consistently influenced the invasivity of p37-positive cells *in vitro* and assay their effect on the invasive behavior of p37-negative cells.
- (3) To analyse single cell collisions between invasive and non-invasive cells using time lapse filming and to examine the effects of anti-p37 Fab on contact inhibition of

locomotion.

(4) To determine the effects of the expression of p37 in cells normally p37-negative on their invasivity *in vitro* and on their contact inhibition of locomotion.

(5) To identify the factor in the culture medium of a metastatic fibrosarcoma cell line that transmitted p37 to other cell lines.

MATERIALS AND METHODS

Tumors, Cell Cultures and Medium

The cell line FS9 is a chemically induced sarcoma from CBA mice and was obtained from Dr. G.A.Dunn, MRC Cell Biophysics Unit, London (Abercrombie, 1979). Since in our laboratory FS9 cells lost their capacity to form tumors in CBA mice, tumors were induced in nude mice by a single subcutaneous injection of a single-cell suspension of at least 10^8 cells. FS9 is a tumor cell-line showing nonreciprocal invasion in the confronted explant assay (Abercrombie, 1979; Paddock and Dunn, 1986).

The L929 tumor cells were obtained from a virus-induced sarcoma of mice and have been well studied (Sandford, 1948). L929 and FS9 cells have the same H-2 haplotype (H-2^K). L929 cells are contact inhibited and were therefore chosen as control cells.

The A375 human melanoma cell line (Kozloski et al., 1984a,b) was a gift of Dr.I.J.Fidler, NCI-Frederick Cancer Research Facility, Maryland. A more metastatic variant designated A375-M was derived from the parent A375 (designated A375-P) by culturing cells derived from experimentally produced lung tumor nodules (Tscrucio et al., 1981).

The PC-3 human prostate adenocarcinoma was also a gift from Dr.I.J.Fidler. The PC-3-P variant represents the less invasive cell line. PC-3M represents the invasive subcolony of the parental PC-3-P (Kozlowski et al., 1984b).

A further human melanoma MM96 (Stephenson et al., 1978) was used. This is highly invasive in the confronted explant assay.

All cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing either 10% heat inactivated fetal calf serum (FS9, L929, A375 and PC-3) or 15% fetal calf serum (MM96).

Selected hybrids (DD9, HE7) secreting monoclonal antibodies (mAbs) against the surface 37kd protein (denoted p37) (Steinemann et al., 1984a,b) were grown in Iscove's modified Dulbecco's medium containing 10% FCS. The culture supernatant was collected and stored frozen for use in western blots, protein A-assays and immunofluorescence studies. The growing hybridomas were injected peritoneally in Balb/c mice to produce ascites fluid.

Large Scale Purification of mAbs

Expanded hybridomas were washed with phosphate buffered saline pH 7.2 (PBS), resuspended to $2 \cdot 10^7$ cells/ml and injected intraperitoneally (0.5ml) into 70 Peristan treated Balb/c mice. Ascites fluid was collected after 10 days, centrifuged and stored frozen.

Large scale purification of mAbs followed a two step procedure: ascites fluid diluted 1:3 with PBS was filled into dialysis membranes and precipitated overnight at room

temperature in Na_2SO_4 (18%w/v). The suspension was centrifuged at $40,000 \times g$, the supernatant decanted and the pellet redissolved in water. After 2 days of dialysis against a K-phosphate buffer (10 mM, pH7.5), the solution was clarified by centrifugation and applied to a DEAE column preequilibrated with phosphate buffer. The yield of protein was estimated by measuring the absorbance at 280 nm assuming the calculation factor of 13.8 absorbance units for a 1% solution. Purified mAbs were stored frozen and later used for affinity immunoadsorption and for digestion to monovalent antibodies (Fab).

Preparation of Monovalent Antibodies by Papain Digestion (Porter, 1959)

Purified DD9 antibodies from ascites fluid were digested with mercuripapain (Sigma) into Fab and Fc fragments. Digestion was done for 16 hr at 37°C with a IgG : enzyme ratio of 100 : 1 in phosphate buffer (0.1 M, pH 7.2) containing cysteine (0.01 M) and ethylene-diamine tetra acetate salt (EDTA 0.002 M) to activate the enzyme. After dialysis against water (48 hr) and sodium acetate buffer (pH 5.5, 0.1 M) fragments were applied to a carboxymethyl (CM)-cellulose column. Elution was done with a gradient from 0.1-0.9 M sodium acetate (pH 5.5). The collected fractions were analysed by SDS PAGE and by measurements of the UV absorption at 280 nm.

Gel System

A discontinuous polyacrylamide electrophoresis system (SDS PAGE) employing 3% polyacrylamide in the stacking gel and 12% or 15% in the separating gel was used to separate proteins according to their molecular weights (Lämmli, 1970). At the end of the run the gels were either stained or western blotted.

Western Blot

Proteins separated by SDS-PAGE were transferred electrophoretically onto nitrocellulose filters (Towbin et al., 1979). The procedure was carried out in a methanol containing blotting medium (0.025M Tris-HCl, 0.193M glycine, 20% methanol (v/v), pH 8.35) at 4°C (Tsang et al., 1983). After treatment with peroxidase-conjugated second antibody the filters were stained for peroxidase activity using 3',3'-diaminobenzidine tetrahydrochloride (DAB) in H_2O_2 .



Protein A-Assay

FS9, L929, A375-P, A375-M, MM96, PC-3-P and PC-3-M cells were washed in PBS, resuspended in 25 μ l aliquots containing 10^6 cells in PBS and pipetted into round bottomed wells of 96-well microtiter plates. Aliquots of 25 μ l DD9 hybridoma supernatant were added to the cells, diluted in log 2 steps in PBS and incubated for 45 min at 4°C. Each sample was tested in triplicate. Cells were washed three times by filling the wells completely with cold PBS and centrifuging of the microtiter plates at 400 g at 4° for 6 min. Supernatants were removed by flicking the plates. The cells were resuspended using a vortex mixer. A 25 μ l sample of 1:5,000 diluted rabbit anti-mouse IgG antiserum in PBS were pipetted into each well and incubated 45 min at 4°C. Cells were washed as described above and finally incubated 45 min at 4°C with 50,000 cpm of 125 I-labelled protein A in PBS. Radioactive supernatants were discarded, and labelled cells were washed three times with cold PBS. The cell solution was transferred from wells into counting tubes and bound radioactivity was measured in a gamma counter.

Immunoadsorption of P37

Purified monoclonal antibody (35 mg) from ascites fluid were incubated overnight with 6.5 ml Affi-Gel 10 (Bio-Rad) following the instructions of the manufacturer. The coupling efficiency was estimated by taking 50 μ l of the supernatant (coupling solution) acidifying this with 950 μ l HCl (0.01 N) and measuring the absorbance at OD 280. Coated beads were washed two times with MOPS (0.1 M, pH 7.4). Uncoated linkers were blocked with 0.7 ml ethanolamine-HCl (1 M, pH 8.0).

Solubilized crude membranes of FS9 cells were incubated with the beads by rotating the tube end-over-end at 4 °C overnight. Then the beads were washed six times by centrifugation at 100 g with PBS, 0.5% Triton X-100. Elution was performed at room temperature with 4 M $MgCl_2$. Fractions of 150 μ l were collected in Eppendorf tubes and analysed by SDS-PAGE. P37 containing fractions were pooled and loaded on a preparative SDS-PAGE system.

Isolation of P37 Using Preparative SDS PAGE for Amino Acid Sequence Analysis

Acrylamide, N,N'-methylenebisacrylamide, N,N,N',N'-tetramethylene diamine, ammonium persulfate, 2-mercaptoethanol, Bromphenol Blue and SDS were purchased from Bio-Rad as electrophoresis-purity reagents, sodium thioglycolate from Sigma and Coomassie Brilliant Blue R-250 (research grade) from Serva. SDS was further purified by crystallization two times from ethanol-water. Dialysis membranes were soaked in 1% $NaHCO_3$ at 60°C for 1 hr, washed with water, soaked in 0.1% SDS solution at 60°C for 1 hr, washed with

water and stored in 0.1% SDS, 0.1% NaN_3 at room temperature.

Proteins prepared by SDS PAGE can be sequenced successfully if a few precautions are taken to minimize aminoterminal blockage of the proteins, cleavage of the proteins into smaller fragments and introduction of contaminants that cannot be removed from the protein prior to sequence analysis. The purity of the reagents used in the procedure is crucial to avoiding a variety of problems, and the reagents (particularly the acrylamide, SDS, and Coomassie Blue) have been purchased from the list above. Samples loaded on gels were not boiled in solubilization buffer, but rather heated to 50-60°C for 10 min. Destruction and degradation by free radicals was prevented by addition of sodium thioglycolate (0.1mM) to the cathode buffer reservoir. The proteins were eluted from gels after visualization by staining with Coomassie Blue (using 0.5% Coomassie in acetic acid-isopropyl alcohol-water, 1:3:6, v/v/v) for 3 min. Destaining (using acetic acid-methanol-water, 50:165:785, v/v/v) was done rapidly with Linsoft tissues placed in the corner of the destaining chamber to adsorb the excess dye. The stained band was sliced away, soaked in water for 2 hr (several changes) and stored at -20°C after draining of the water.

Electrophoretic elution: Gel slices were soaked in elution buffer (0.1%SDS in 0.05M NH_4HCO_3) for 5 min. The gel pieces were transferred into an electrophoretic elution cell (Hunkapillar et al., 1983) fitted with dialysis membranes (exchange limit 15,000 dalton) covered with soaking buffer (2% SDS in 0.4 M NH_4HCO_3 , 0.1% DTT). After soaking 3 hr the gels were overlaid with elution buffer, and proteins eluted in an electrode chamber at 50 V dc for 12-16hr. After the elution has proceeded the elution buffer was replaced in the tank with dialysis buffer (0.02% SDS in 0.01M NH_4HCO_3) and elution-dialysis was continued at 80 V dc for another 20-24hr. The protein solution was transferred to an Eppendorf tube, and a small sample was separated for SDS PAGE analysis.

Mycoplasma Elimination with BM-Cycline

The respective cell lines were transferred to 10 ml medium containing 40 μ l BM-Cycline (Boehringer Mannheim, Biochemica). Cells were left to grow for three days, in the usual way with no other medium changes. At the end of the third day the medium was removed as completely as possible and 10 ml fresh medium containing 40 ml BM-Cycline 2 was added. Culturing was continued for 4 days. The cycle was repeated beginning on day 7 of the treatment. The cultures were checked with DAPI (4,-6-diamidino-2-phenyl-indoledihydrochloride) every week to monitor the mycoplasma disappearance. Normally mycoplasmas were absent after a week but as this was only an optical determination the treatment was continued for a further two weeks.

Time Lapse Recording of Cell Behavior

Primary explants of chick heart ventricle from 7-9 day-old embryos were cultered in DMEM at 37°C. Outwandering cells were trypsinized two days later and mixed with a cell suspension of tumor cells. The mixed cultures were seeded on acid- washed cover slips and were allowed to settle for 1-2 hr before use. Cell behavior was recorded at 37°C using Zeiss Universal phase contrast microscopy equipment. Kodak Plus-X was used in a Bolex H-16 camera at frame intervals of 30 sec. determined by a Wild-Paillard Variotimer. The selection of the cell analysis was governed by the requirements that they should remain within the imaged area for at least an hour and that either they should be free from contact with other cells or they should collide with only one other cell (Dunn et al., 1982; Paddock and Dunn, 1986).

Data Storage

OLINES, a program designed by Dr A. Brown and Dr G. Dunn (MRC Biophysics Unit, London), was used to store outlines, drawn on a pitpad. For each cell collision the path of a series of points, representing the sequential position of a nucleus at 1 min intervals, was fed into a PDP 11/44 computer using a digitizing tablet (Summagraphic pitpad).

Data Analysis

Paddock and Dunn (1986) developed a system to analyse the changes in motion of pairs of colliding cells. For a sample of collisions carried out under the same conditions (for example with and without antibody) x-means were calculated for both FS9 cells and chicken heart fibroblasts. This value represents the scalar difference between how far the cell has actually progressed in the direction it was moving before the collision and how far it would have progressed had there been no change in motion. The more negative the x-means, the higher the contact inhibition of locomotion.

Confronted Explant Test

Round acid-washed cover slips were placed in 24-well microtiter plates. Heart ventricles of 7-9 day old chicken embryos were cut into small pieces (0.5mm^3), transferred into fresh medium (DMEM, 10%FCS) and then placed on the cover slips. Tumors grown either in nude mice prepared in the same way as chicken heart ventricles or, alternatively, clumps of cultured tumor cells grown to high density were placed on the cover slips 1 mm distant from the chicken heart explants. Confronted explants were then incubated at 37°C to allow the cells to migrate outwards from the explant. Fabs were included in the culture medium. Twelve to 24hr after the cells from the two explants had made their first contact, the cultures were fixed with 3% formaldehyde. From measurements of fixed cultures, "between/side" ratios and invasion indices were calculated (Abercrombie et al., 1976; Stephenson, 1982) to compare migration distances achieved by a given population between the confronting explants with migration distances achieved by unconfronted cells of the same population moving into free space.

Immunocytochemistry

Cells were grown overnight on acid-washed cover slips in DMEM containing 10% FCS. After a rinse with PBS followed by fixation with freshly prepared formaldehyde (3% in PBS, 15min), the cells were washed two times 15 min each with PBS and incubated in hybridoma supernatants (containing 100-200 ng/ml mAbs) for 1h at 37°C . After briefly washing with PBS (two times, 15 min) FITC labelled rabbit anti-mouse IgG (1:100) (Nordic, Immunological Laboratories) in PBS containing 1% BSA were applied for 30 min at 37°C . After washing with PBS as described above, cover slips with attached cells were placed upside-down onto a drop of 70% glycerol in PBS. p-Phenyldiamine was used to prevent fading. Fluorescence was monitored using a Zeiss Universal microscope.

RESULTS

1. Preparation of Monoclonal Antibodies and Fab Fragments

High titers of monoclonal antibodies were prepared for estimating p37 levels on cells with the protein A test and for affinity chromatography purification of p37.

Large amounts of monoclonal antibodies were produced either by culturing hybridoma cells *in vitro* or growing them in mice. The monoclonal antibody was secreted and accumulated in the spent medium of the cultured cells and in the serum and the body fluid of the tumor bearing animals. The concentration of the monoclonal antibody in the spent medium was of the order of 10-50 µg/ml. The concentration of the antibody in the body fluid of tumor bearing animals can be 1000 times higher.

We injected 2×10^7 DD9 hybridoma cells from cultures or a same number of cells from centrifuged ascites fluid into the peritoneal cavity of a Balb/c mouse. The animals were histocompatible with the hybrid clone being injected. Ascites fluid was removed from 60 male Balb/c mice about 10 days after the inoculation. The antibodies were purified with sodium-sulfate precipitation and DEAE-chromatography. The yield of antibodies was determined from optical densities at 280nm. The fractions of the DEAE column were analysed by SDS PAGE (Fig. 1).

The pooled fractions of purified IgG were digested with mercuripapain and the antibody fragments obtained then isolated by chromatography on carboxymethyl (CM)-cellulose (Fig. 2a). Fabs were obtained in a single protein peak as indicated by the profile from UV absorption measurements at 280nm. Fc fragments could not be detected with UV absorption and SDS PAGE. From the 20 mg of digested mouse IgG 14 mg purified Fab's were collected. The purity was monitored on SDS PAGE (Fig. 2b).

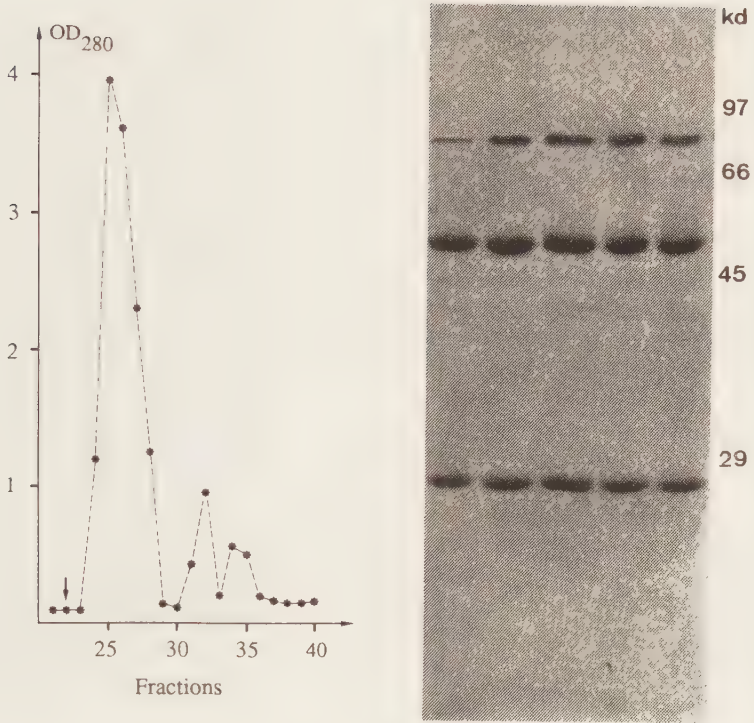


Fig. 1

Purification of IgG.

a) DEAE (DE 52, Whatman)-cellulose chromatography of precipitated monoclonal antibody DD9 from ascites fluid. The IgG was eluted with a 200 ml gradient of 10 mM - 150 mM phosphate buffer, pH 7.4. The column was initially washed with 100 ml of 10 mM phosphate buffer. The arrow indicates where the salt gradient commences. Seven ml fractions were collected. 70 mg purified IgG were obtained from the fractions 23-29. The monoclonal antibody was eluted prior to the normal mouse IgG's.

b) SDS PAGE of fractions 24 - 28. The gel was silver-stained.

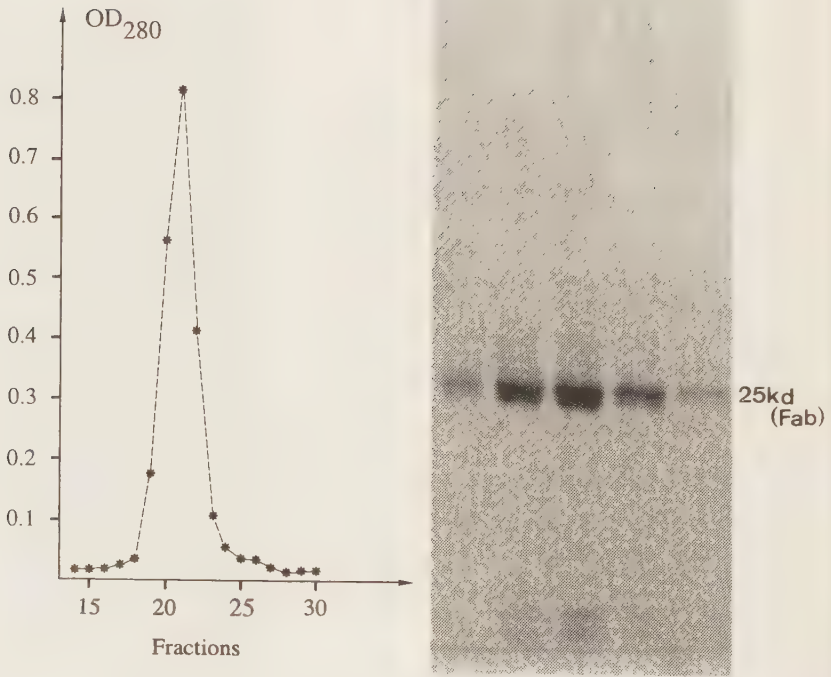


Fig. 2

Preparation of Fab.

- a) Carboxymethyl (CM 52, Whatmann)-cellulose chromatography of 20 mg papain-digested monoclonal antibody (DD9). The Fab was eluted with a linear 200 ml gradient of 0.1 M to 0.9 M sodium acetate buffer (pH 5.5). The column was initially washed with 40 ml 0.1 M sodium acetate buffer. The arrow indicates where the gradient commences.
- b) SDS PAGE of fractions 19-23 obtained by CM-cellulose chromatography.

2. Purification and N-terminal Sequence of P37

One of the aims was to clone the gene for p37, use this to transform cells and select p37-positive transformants. The effects of p37 expression on the invasive behavior of such transformed cells was then to be studied.

Coupling of DD9 IgG to Affi-Gel 10

Aqueous coupling of 36 mg purified IgG from ascites fluid to 6.5 ml Affi-Gel 10 was carried out in a volume of 25 ml overnight at 4 °C. The concentration of soluble (unbound) proteins remaining in the coupling and wash buffers was estimated by measuring optical density at 280 nm. A coupling efficiency of 82% was estimated. 29.5 mg IgG were coupled to the linkers of Affi-Gel 10.

Immunoaffinity Chromatography of P37 with Immobilized DD9 IgG

We purified p37 by immunoaffinity chromatography. DD9 IgG were immobilized on Affi-Gel 10 beads. The FS9 sarcoma cells (5×10^6) were solubilized in 1% Triton X-100 and incubated with the Affi-Gel 10 beads as described in "Materials and Methods". The bound proteins were eluted with 4 M $MgCl_2$ and fractions of 150 μ l were collected. One μ l of every fraction was loaded on to SDS PAGE and proteins detected by silver staining to monitor the elution profile. (Fig. 3)

Isolation of P37 with Preparative SDS PAGE

The fractions containing p37 eluted from the Affi-Gel 10 beads were pooled and loaded onto a preparative gel system as described in 'Materials and Methods'. Sixty preparative gels were run and the proteins were visualized by staining with coomassie blue. The p37 band was sliced from the destained gel. Proteins were eluted from gel slices as described in 'Materials and Methods'. Eluted proteins were precipitated with ethanol (protein solution : ethanol , 1 : 9 , v : v) at -20 °C to remove excess SDS. Subsequently, the proteins were lyophilized at -60 °C and resuspended in H_2O . Three different unknown concentrations of the protein were run on SDS PAGE together with a series of known concentrations of bovine serum albumine (BSA) to estimate the quantity of p37 (Fig. 4). We estimated a total amount of 60 - 120 μ g p37 were isolated. This value indicates that there are at least $2-4 \times 10^5$ molecules per cell.

About 30-60 μ g of the isolated p37 were put aside for amino-acid sequencing the N-term.

A CNBr cleavage with 20 - 40 µg of p37 for amino-acid sequencing of internal peptides was not successful and peptides could not be eluted from the HPLC-column.

N-terminal Sequence Determination

We purified p37 to homogeneity using immuno-affinity chromatography and preparative SDS PAGE. Microsequencing, using a gas-phase sequenator, yielded the first 29 amino acids of its N-term. The sequencing was done in collaboration with Prof. Boehlen, University of Zurich and Dr. R. Wettenhall, La Trobe University, Melbourne. No signal was determined in the first cycle (X), suggesting Cys may be present since it cannot be detected with the methods used. Amino acid 19 could not be unambiguously determined. The sequence obtained was:

N-term	X	Ser	Asn	Thr	Gly	Val	Val	Lys	Gln	Glu	Asp	Val	Ser	Val
	Ser	Gln	Gly	Gln	His Trp Lys	Asp	Lys	Ser	Ile	Thr	Phe	Gly	Val	Asn Pro

At this point we discovered that p37 could be transmitted to other p37-negative cells using FS9 cell culture supernatant. We were thus able to test the effects of gaining p37 on cell invasive behavior without having to transform cells with the p-37 gene. (Note: the gene has been subsequently cloned and sequenced. See discussion).

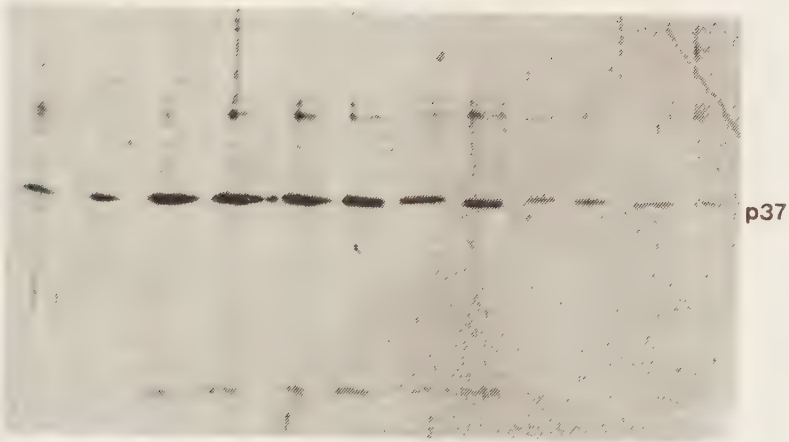


Fig. 3

Purification of p37 by immunoaffinity chromatography.

Purification of p37 by immunoaffinity chromatography of solubilized FS9 proteins on Affi-Gel 10 beads. Purification was monitored by SDS PAGE of bound proteins. Small amounts of IgG were occasionally also eluted from the beads.

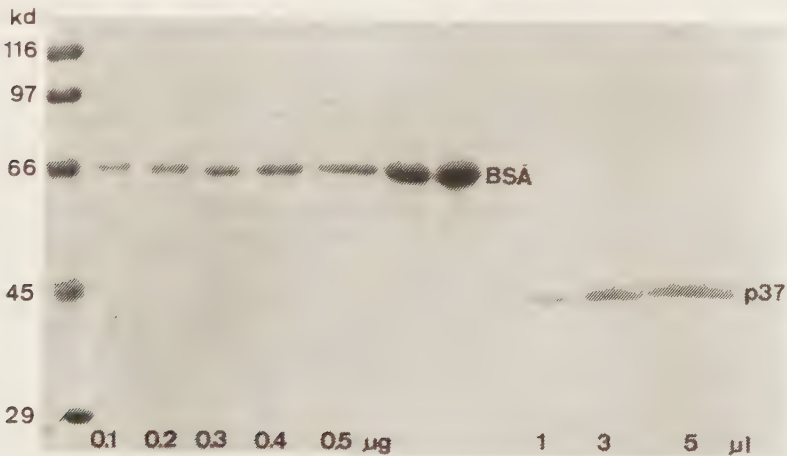


Fig. 4

Estimation of the concentration of purified p37.

Coomassie blue stained SDS PAGE of purified p37 (1, 3, 5, μ l) and BSA (0.1, 0.2, 0.3, 0.4, 0.5, 1, 2, μ g). One μ l of p37 corresponds to 0.1 - 0.2 μ g BSA. Coomassie blue binds stoichiometrically to proteins.

3. A Variety of Cell Lines Carry the P37 Antigen

Cell lines whose metastatic activity *in vivo* has been studied were screened for the presence of p37. Correlations between the levels of p37 and metastatic behavior were sought.

Screening of Different Cell Lines for the Presence of P37

Because the presence of p37 is associated with the invasive behavior of FS9 cells (Steinemann et al., 1984 a b) we screened a variety of cell lines, including human tumors, for the presence of p37. In particular, we sought cell lines where variants with high and low metastatic potential were available (Talmadge et al., 1982; Kozlowski et al., 1984a,b; Vollmers et al., 1983, 1984).

Kozlowski et al., (1984) have isolated both highly metastatic (A375 M) and low metastatic (A375 P) subclones from the human melanoma cell line A375. (Metastatic activity was tested in athymic nude mice). We solubilized crude membranes from these subclones in 1% Triton X-100 and separated the proteins using SDS PAGE. Western blotting with a monoclonal antibody specific for p37 (DD9) demonstrated that p37 was present on the metastatic A375 M cells but not on the non-metastatic A375 P cells (Fig. 5).

The second human melanoma cell line MM96, which has been extensively tested in Abercrombie's confronted explant test by Stephenson et al., (1978) and Stephenson (1982), and is highly invasive was also screened. These cells were positive for p37 in western blots (Fig. 6)

However, the non malignant human prostate adenocarcinoma, PC 3-P, and its malignant subclone, PC 3-M (Kozlowski et al., 1984), both gave a p37-positive signal in western blots (Fig. 7).

Metastatic and non-metastatic variants of the mouse melanoma cell line B16 were also available. The highly metastatic variant (B16 F10) was isolated by multiple passages through the lung and other organs *in vivo* (Brunson et al., 1978; Fidler, 1975; Vollmers et al., 1983) We found that neither B16 F10 nor a cell line selected for low metastatic potential (B16 F1) carried p37 (Fig. 8).

No p37 was detected in western blots with 3T3 mouse fibroblasts (Fig. 9). RAT-1 fibroblasts, which have more or less the same invasive behavior in Abercrombie's confronted explant assay as 3T3 cells, did show a p37-positive reaction on western blots.

Comparison of the Amounts of P37 in Different Cell Lines

Several immunoassays depend on the capacity of the protein A from *Staphylococcus aureus* to bind selectively to the Fc fragment of immunoglobulins. Since only certain subclones of mouse IgG are recognized by protein A, we also tested the monoclonal antibody against p37 from hybridoma strain DD9 and the second polyclonal rabbit anti-mouse antibody (Nordic immunological laboratories) for their capacity to bind protein A. DD9 hybridoma supernatant, purified ascites fluid of DD9 and polyclonal rabbit anti-mouse antibody were coated onto flexible microtiter plates in series of ten-fold dilutions. Subsequently, iodinated protein A was incubated with the antibodies, and bound radioactivity was gamma-counted. Fig. 10 depicts the ability of protein A to bind to monoclonal antibody DD9 and polyclonal rabbit anti-mouse antibodies.

To compare the amount of p37 in cell lines, aliquots of 10^6 cells were pipetted into a well of a microtiter plate, then incubated with the monoclonal antibody against p37 (diluted in log 2 steps) and, to increase the sensitivity of the assay, incubated with a second rabbit anti-mouse antibody (see 'Materials and Methods'). The cells were gamma counted after incubation with iodinated protein A (50,000 cpm per well). Every cell line was assayed in triplicate and standard deviation was estimated as less than 10% of the mean. Since we observed that L929 cells also gave a weak signal in the protein A assay with a prozone effect, we used 7 day cultured embryonal chicken heart fibroblasts (CHF) as negative controls (Fig. 11a). To estimate the unspecific binding of rabbit anti-mouse antibody and iodinated protein A, the assay was performed with and without monoclonal antibody against p37 (Fig. 11a+b)

Results of the protein A assay performed on FS9 and L929 cells were consistent with those of Steinemann et al., (1984b). FS9 cells had about six time more antigenic determinant which bound DD9 specifically. However, a p37-positive signal was never detected on western blots of L929 cells, although these cells bind significantly more antibody than CHF (Fig. 12).

When the human melanoma lines A375 M and A375 P were tested in the protein A assay (Fig. 13) with the same DD9 supernatant and the same dilution of DD9 ascites fluid, A375 M bound much higher amounts of antibody than the non-metastatic subclone A375 P. The binding capacity was higher than for FS9 cells. This was expected as the A375 cells are larger than FS9 cells and the assays were carried out with the same number of cells for all cell lines (10^6 cells / well). A375 P and L929 cells bound similar levels of DD9 antibody. Monoclonal antibody DD9 failed to bind to nitrocellulose filters carrying plasma membrane proteins of either A375 P or L929 cells.

The metastatic and non-metastatic variants of the human prostate adenocarcinoma PC 3 were both p37-positive. Using protein A assays we found that PC 3-P bound slightly more p37-specific antibody than PC 3-M (Fig. 14). Binding was comparable to that observed with FS9 cells. FS9 and PC 3 cells were of similar size.

The highly invasive p37-positive human melanoma MM96 has a surface about 3 times larger than FS9 cells. Hence, it was not surprising to find that these cells had the highest binding capacity of all lines tested in the protein A assay (Fig. 15).

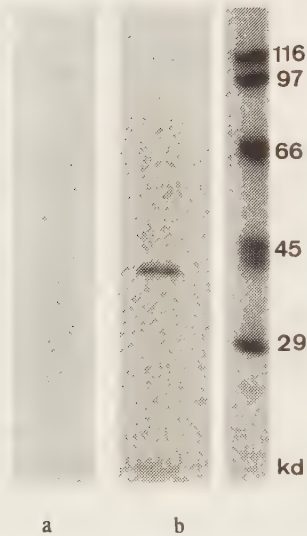


Fig 5



Fig 6

Fig. 5

Western blot of (a) A375 P cells and (b) A375 M melanoma cells. Proteins from crude membranes were separated by 12% SDS PAGE and transferred electrophoretically to nitro-cellulose filters. Immunodetection of p37 was performed with the monoclonal antibody DD9.

Fig. 6

Western blot of crude membrane proteins from the human melanoma MM96 (a) and FS9 (b). Proteins were separated on 12% SDS PAGE. Immunodetection was performed with the monoclonal antibody DD9.

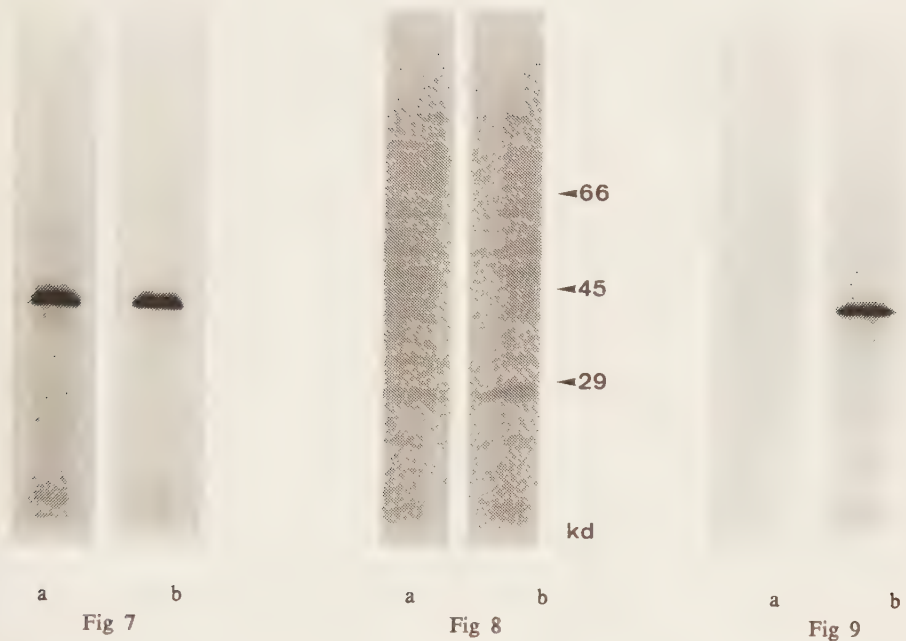


Fig. 7

Crude membranes of human prostate adenocarcinoma cell lines PC 3-P (a) and PC 3-M (b) were separated with 12% SDS PAGE and transferred electrophoretically to nitrocellulose filters. Immunodetection of p37 was performed with DD9.

Fig. 8

Crude membranes of the mouse melanoma cell lines B16 F1 (a) and B16 F10 (b) were separated with 12% SDS PAGE and then transferred to nitrocellulose filters. No p37 was detected after incubation with monoclonal antibody DD9.

Fig. 9

Western blots of a) NIH 3T3 mouse fibroblasts and b) RAT-1 fibroblasts. Immunodetection was performed with DD9. P37 was detected on the RAT-1 cells.

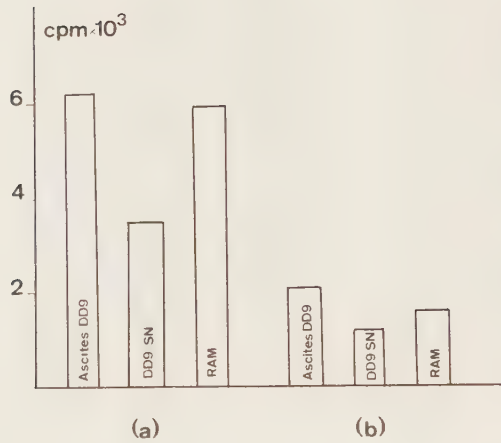


Fig. 10

Affinity of the DD9 monoclonal antibody and rabbit anti-mouse antibodies for protein A. Microtiter plates were coated with 25 μg (a) or 2.5 μg (b) monoclonal antibody from ascites fluid, rabbit anti-mouse antibody or an unknown concentration of antibody from hybridoma supernatant (SN). They were then incubated with iodinated protein A.

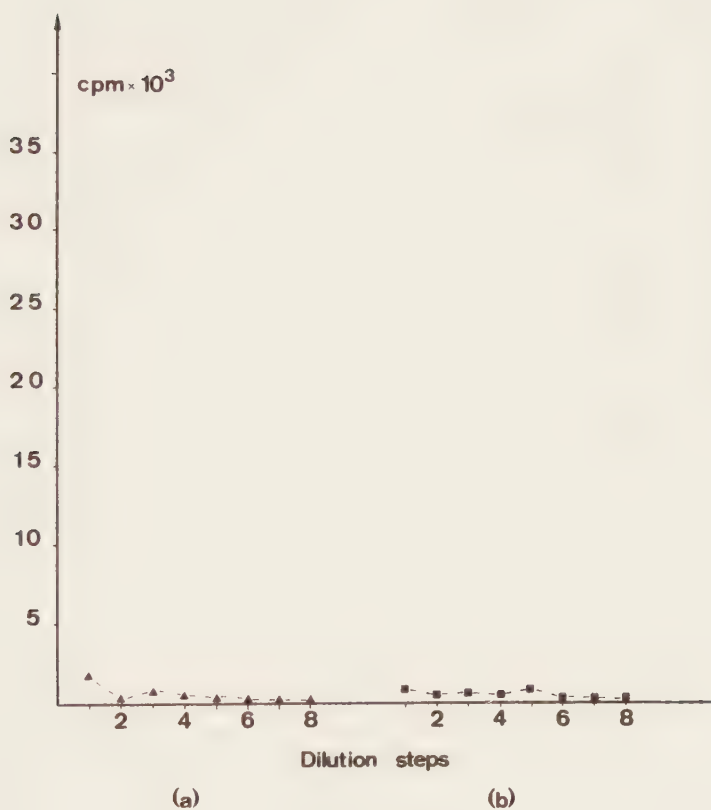


Fig. 11

The binding of DD9 monoclonal antibody and an unspecific rabbit anti-mouse antibody to cells. Iodinated protein A was used to determine the levels of antibody bound.

(a) Binding of the DD9 hybridoma supernatant to chicken heart fibroblasts (CHF). The supernatants were diluted in log 2 steps.

(b) Binding of unspecific rabbit anti-mouse antibody immunoglobulin G (IgG) and iodinated protein A to FS9 cells in the absence of antibody directed against FS9 membrane antigens.

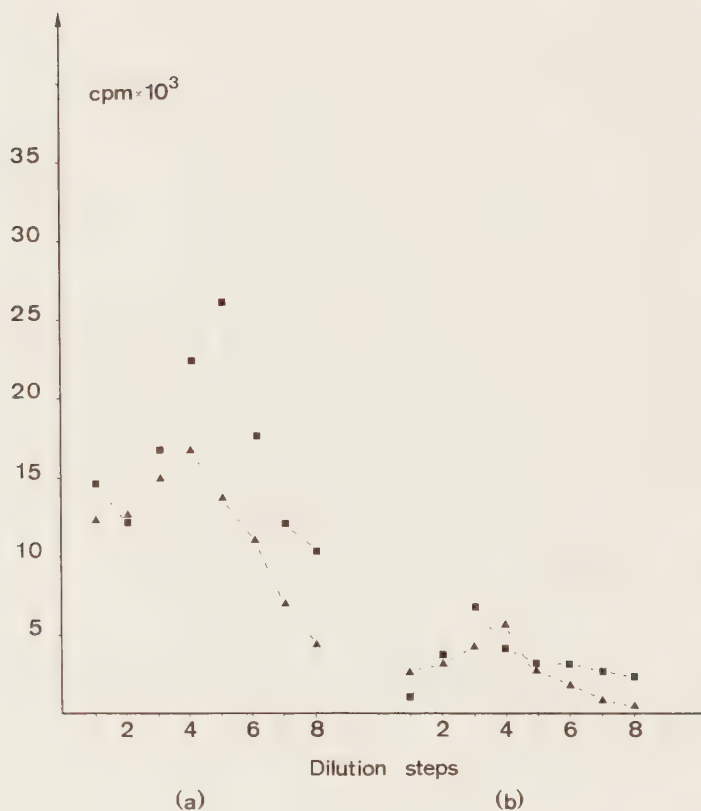


Fig. 12

Binding of the DD9 hybridoma supernatant (▲) and the DD9 ascites fluid (■) to FS9 cells (a) and L929 cells (b). The antibody was diluted in log 2 steps. The assay was performed using protein A as described in 'Material and Methods'. Points in the titration curves represent means of triplicate experiments after subtraction of the values obtained with the negative controls.

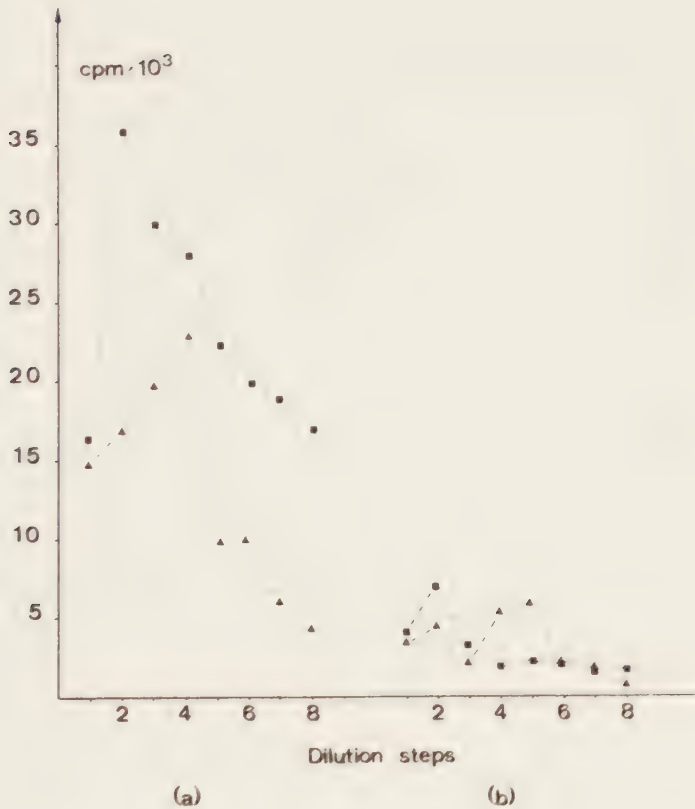


Fig. 13

Binding of the DD9 hybridoma supernatant (▲) and the DD9 ascites fluid (■) to A375 M cells (a) and A375 P cells (b). The antibody was diluted in log 2 steps. The assay was performed using protein A as described in 'Materials and Methods'. Points of the titration curves represent means of triplicate experiments after subtraction of the values obtained in negative controls.

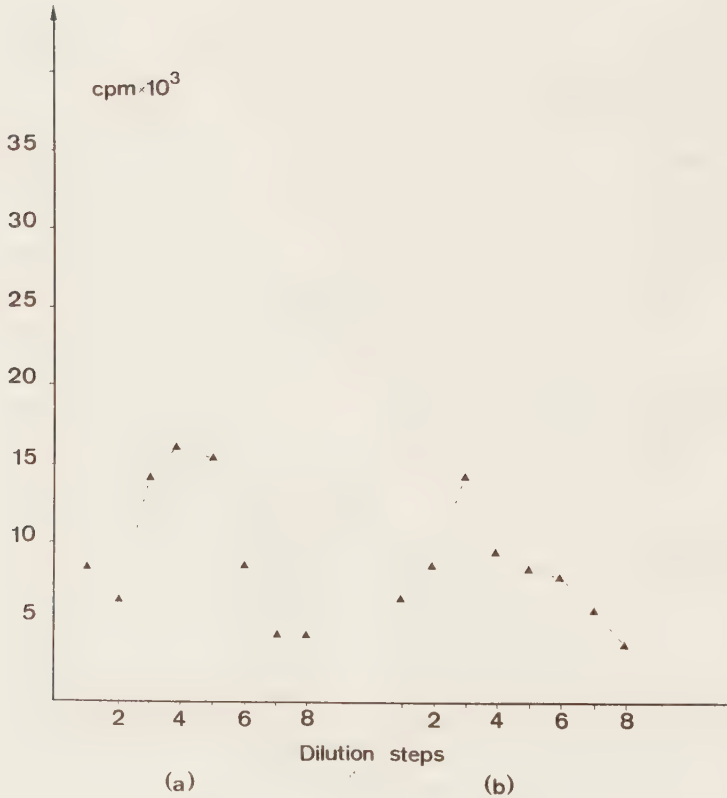


Fig. 14

Binding of the DD9 hybridoma supernatant to PC 3-P cells (a) or PC 3-M cells (b). The antibody was diluted in log 2 steps. The assay was carried out using protein A as described in 'Material and Methods'. Points in the titration curves represent means of triplicate experiments after subtraction of the values obtained in negative controls.

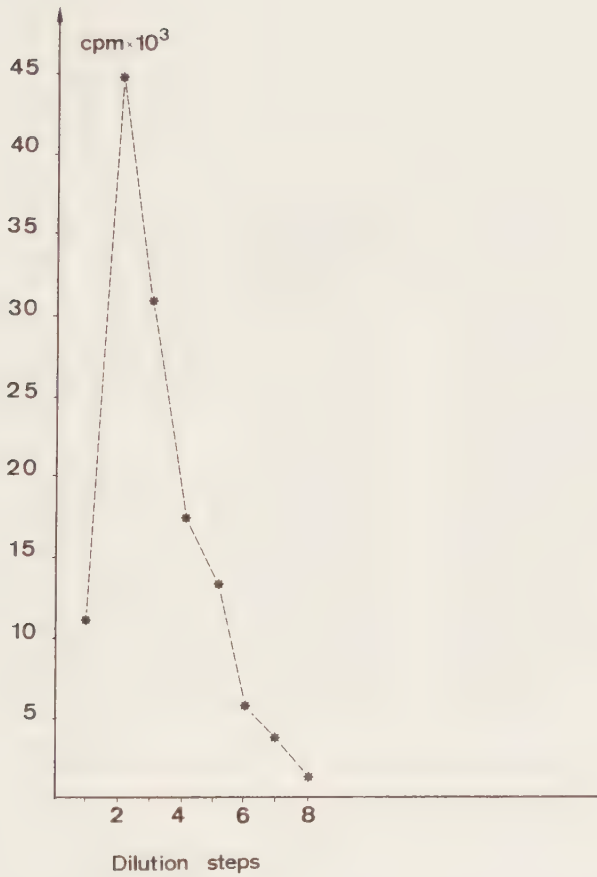


Fig. 15

Binding of the DD9 supernatant to human melanoma cells MM96. Antibody was diluted in log 2 steps. The assay was performed using protein A as described in triplicate experiment after subtraction of the values obtained in negative controls.

4. P37 is Transmitted Between Cell Lines

P37 is inducible in L929 cells. Immunofluorescence studies demonstrated that the L929 cells expressed p37 eight days after an inoculation with sterile filtered (filtered through 0.22 μm filters) FS9 culture supernatant (Dr. H. Binz, personal communication).

This observation suggested that either infectious agents, such as viruses and mycoplasmas, or cellular factors, such as autocrine regulators, may be responsible for the appearance of p37 in L929 cell cultures. We wished to study this phenomenon in detail, using western blotting to screen for the appearance of p37 in infected/induced cell cultures.

It was possible to transmit p37 to L929 cells (now designated L929t, Fig 16), to A375 P cells (subsequently designated A375 Pt), to B16 cells (subsequently designated B16t), or to 3T3 cells (subsequently designated 3T3t) with sterile filtered FS9 culture supernatant. P37 appeared in the cell cultures after 7 to 10 days (Fig. 17). P37 could be similarly induced in all cell lines subsequently tested.

Treatment of L929 cells with the culture supernatant of L929t cells also resulted in the appearance of p37 in the L929 cell cultures (Fig. 18). Similarly, when sterile filtered culture supernatant of A375 M cells was added to L929 cells, p37 appeared in the latter cultures after 7-10 days.

FS9 tumors cultivated in nude mice lost p37. Four week old athymic Balb/c nude mice were given a single subcutaneous injection of 1×10^6 FS9 cells and were killed 2 weeks later. A capsular tumor colony was harvested at the injection site. P37 was not detected in solubilized cells from this tumor (Fig. 19). However, indirect immunofluorescence staining, carried out by Steinemann et al., (1984b), indicated that p37 is expressed on tumor explants under the conditions of Abercrombie's confronted explant test.

In an attempt to destroy putative membraneous infectious agents, we treated the FS9 culture supernatant with 0.1% deoxycholate. It was not possible to detect any p37 on L929 cells cultured with the deoxycholate conditioned medium (1:100 diluted) even after the cells had grown for 3 weeks in this medium (Fig. 20). Cells were not damaged by the deoxycholate (0.001%) and grew normally. These results suggested either viruses or mycoplasmas as an inducer or carrier of p37.

Retroviruses could be isolated from FS9 cells using sucrose density gradient centrifugation. The viruses isolated in this way did not contain p37 and treatment of L929 cells with them did not result in the appearance of p37 (Meier, 1987).

The presence of mycoplasma infection in cell cultures was determined using the DAPI DNA staining technique. Mycoplasma showed up clearly in the fluorescence microscope (Fig. 21a). Most of the cell lines we studied were positive for mycoplasma, irrespective of their invasive behavior or presence of p37. Treatment of cell lines with anti-PPLO agent (Gibco, 6000 mcg / ml) failed to eliminate mycoplasmas. During the course of this work a new treatment to kill mycoplasmas became available (BM-cycline, Boehringer Mannheim). Although previous treatments had had no effect on p37 levels and

mycoplasma contamination, addition of BM-cycline to cells resulted in the disappearance of mycoplasma, as monitored by DAPI staining (Fig. 21b). Moreover, western blotting showed that p37 disappeared from cultures treated with BM-cycline (Fig. 21b). The conclusions to be drawn from these results are that p37 is either a mycoplasma protein or mycoplasmas have the capacity to induce p37 on the cells.

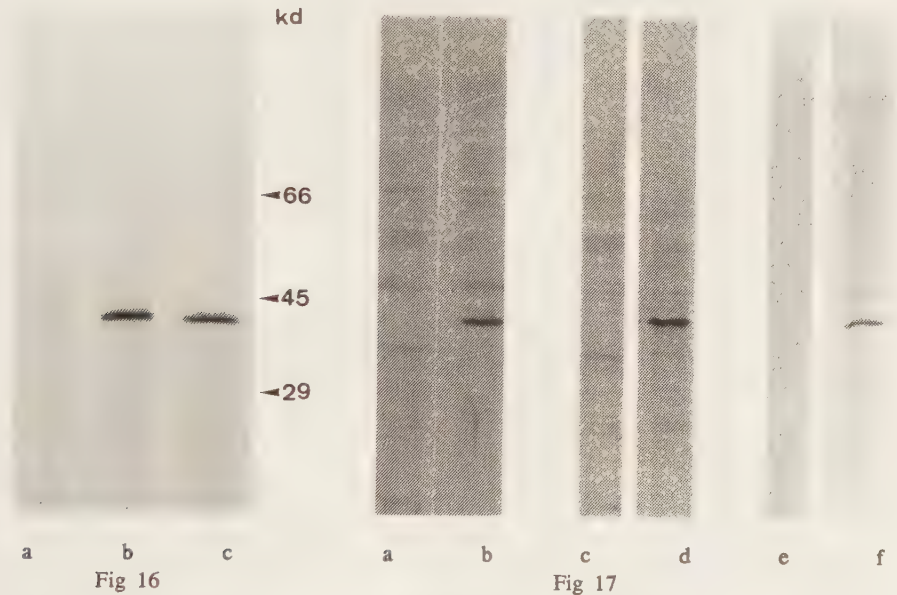


Fig. 16

Induction of p37 in L929 cells by FS9 cell culture supernatant.

Western blot of (a) L929 proteins, (b) FS9 proteins and (c) proteins of L929 cells following cultivation for 8 days in medium conditioned with FS9 culture supernatant (1:100). Immunodetection was performed with DD9 hybridoma supernatant.

Fig. 17

Induction of p37 in various cell lines by FS9 cell culture supernatant.

Cell lines were treated with a 1:100 diluted FS9 cell culture supernatant and, after incubation for 7-10 days, proteins were separated on SDS PAGE, electroblotted and the presence of p37 detected with the monoclonal antibody DD9. a) A375 P cells, untreated, b) A375 P treated (A375 Pt), c) B16 F1 cells, untreated, d) B16 F1 treated (B16 F1t), e) 3T3 cells, untreated, f) 3T3 fibroblasts, treated (3T3t)



Fig 18

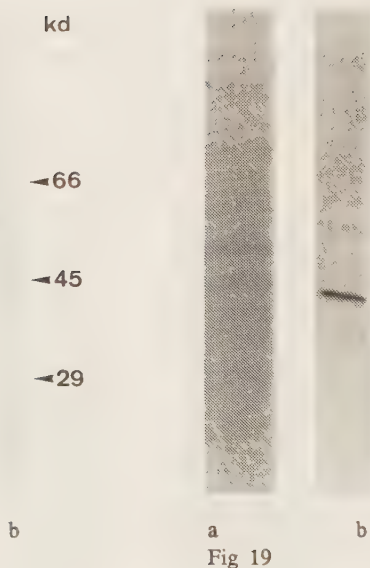


Fig 19



Fig 20

Fig. 18

The appearance of p37 in L929 cells following incubation with culture supernatant from L929 cells that had previously been treated with FS9 culture supernatant and contained p37 (L929t).

The presence of p37 was detected by western blotting. L929 cells grown for 8 days in culture medium (1:100 diluted) obtained from L929t cells (a) and L929 cells grown for 8 days in normal medium (b).

Fig. 19

The effect of growth in mice on the p37 levels in FS9 cells.

FS9 tumor cells were grown for 2 weeks *in vivo* in athymic nude mice. No p37 was detectable. As a control FS9 cells from *in vitro* cell cultures were used. Western blots were used to assay for p37

FS9 tumors removed from mice (a) and FS9 cells, from *in vitro* cell cultures, used to inject the mice (b).

Fig. 20

The effect of deoxycholate treatment on the p37 inducing activity of FS9 culture supernatant.

L929 cells were grown for 1 week (a) or 3 weeks (b) in 1:100 diluted FS9 culture medium that had previously been treated with 0.1 % deoxycholate. Western blots were used to assay for p37.

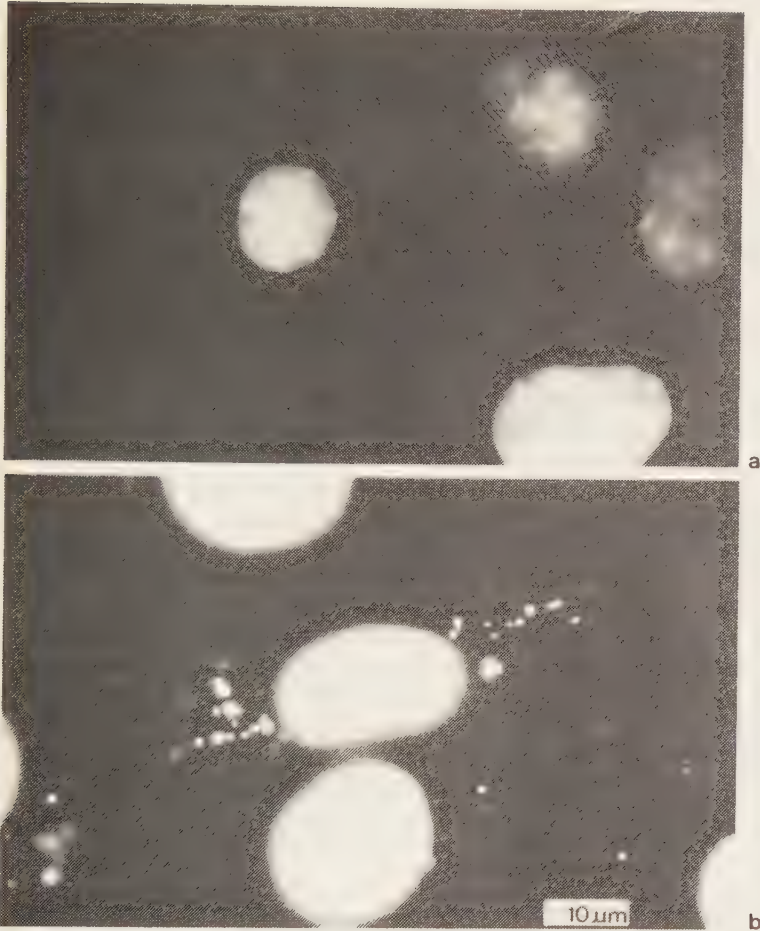


Fig. 21

Detection of mycoplasma in cell culture.

FS9 cells were stained with DAPI and examined in a fluorescence microscope. The large stained organelles are nuclei, the small points are mycoplasma. The respective western blots, showing the presence or absence of p37, are also included.

(a) FS9 cells pretreated with BM-cycline. (b) Untreated FS9 cells, stained with the DNA stain DAPI and western blotted. Immunodetection was performed with DD9 hybridoma supernatant.

5. Removal and Addition of P37 to Cells Profoundly Modifies their Invasion *in vitro*

Since p37 could be induced in other cell lines or eliminated from FS9 cells, we wished to determine whether the appearance of the protein could influence the *in vitro* invasivity of these cells as measured by Abercrombie's confronted explant assay.

An invasion index could be calculated for every confronted explant. The invasion index is defined as the distance of the cell movement between the explants (ie. where contact between the two cell populations is occurring) divided by the distance the cells move at the sides of the explants (where no interpopulation contact occurs). Fixed explants were used to measure these distances. The invasion index is near zero for a cell type totally stopped by the second cell type and 1.0 for a cell population totally unimpeded. Contact promotion, as described by Paddock and Dunn (1986), implies an invasion index greater than 1.0.

FS9 cells invade CHF but not vice versa (non reciprocal invasion) and the invasion indices calculated were consistent with those described by Steinemann et al., (1984).

The FS9 index (0.7725), estimated from 24 confronted explants, demonstrated the high invasive potential of these mouse sarcoma cells (table 1). On the other hand, the chicken heart fibroblasts gave only an index of 0.2075. Standard deviation and variance were calculated in table 1. The effect of Fab derived from monoclonal antibody DD9 directed against p37, was apparent in Abercrombie's confronted explant system at concentrations as low as 0.1 mg/ml. The invasion of CHF by FS9 cells in the area between the explants was strongly inhibited (invasion index: 0.3600). When Fab derived from control IgG or from anti-L929 plasma membrane rabbit antiserum preabsorbed with FS9 cells was used, no effect on FS9 cell invasivity was observed (Steinemann et al., 1984a+b). The non-invasive control cell line L929 had an invasion index of 0.2450 estimated from 15 explants. This was about the same quotient as calculated from non-transformed chicken heart fibroblasts. There was no significant change in contact inhibition following addition of 0.1 mg/ml Fab from DD9 to L929 cells in the confronted explant system (invasion index: 0.2328; table 1).

Treatment of FS9 cells with BM-cycline resulted in the loss of p37. Cells treated in this way (FS9mf) had an invasion index of 0.6020. The addition of specific Fab (0.1 mg/ml) directed against p37 to p37-free FS9 cells reduced the index significantly (to 0.3906). We also examined the effect of unspecific Fab on the invasive behavior of p37-free FS9 cells. No significant modifications of the invasion index occurred (table 1).

We compared the invasivity of 3T3 and 3T3t cells. The presence of p37 on 3T3 cells modified the invasion index. 3T3 cells had an invasion index of 0.5090 calculated from 20 confronted explants. They exhibited some non-reciprocal invasion but were clearly less invasive than FS9 cells although more invasive than L929 cells. 3T3 cells grown in medium conditioned with sterile filtered FS9 culture supernatant expressed p37. When confronted with CHF these 3T3t cells demonstrated a much higher invasion index (0.7788)

than the normal 3T3 cells (table 1). The relatively large standard deviation indicated that the invasive behavior of both 3T3 and 3T3t cells varied considerably. When DD9 Fab (0.1 mg/ml) was added to the confronted explant assay, the invasive index of the 3T3t cells was greatly reduced, namely to 0.3360 (table 1). Five assays were carried out. Unexpectedly, when Fab was added to normal 3T3 cells (ie. lacking p37), their invasive index was reduced to 0.1767 (table 1). This was even lower than the invasion index of CHF. Nevertheless, the data was derived from only 3 tests and a statistical analysis of the data is presented below.

The RAT-1 cells expressed p37 (see earlier). The invasion index computed from 20 confronted explants (0.4425) was comparable with that of 3T3 cells. As expected, RAT-1 fibroblasts grown for 10 days in the inducing medium (normal medium conditioned with sterile filtered FS9 culture supernatant), showed no significant increase in p37 level. The invasion index of RAT-1t (0.5070) did not differ much from that of RAT-1 cells (0.4435). Once again, a dramatic reduction in the invasion index (to 0.2800) resulted from including 0.1 mg/ml anti-p37 Fab in the assay (table 1).

We carried out a statistical analysis of the invasion indices shown in table 1 to determine the significance of any modifications using a t-test for two independent means. Fab-treated cells were compared with untreated cells and means was robust enough for all the pairs to compare. In the test t is the test statistic, $t_{0.05}$ is the confidence level for 95% and $t_{0.01}$ the confidence level for 99%. The higher the t in absolute value, the lower the associated error-probability value and the greater the evidence against the null hypothesis that the two true means are equal. That is, if the error probability is low (traditionally less than 5%) then the observed difference in means is said to be statistically significant.

Invasion index				
cell line	n number of confronted explants	x average of invasion index	s standard deviation of invasion index	s ² variance of invasion index
FS9	24	0.7725	0.1601	0.0256
CHF	24	0.2075	0.0727	0.0053
FS9 +Fab	18	0.3600	0.1433	0.0205
CHF +Fab	18	0.2526	0.0799	0.0064
L929	15	0.2450	0.1496	0.0224
CHF	15	0.2153	0.0623	0.0033
L929 +Fab	11	0.2382	0.1298	0.0169
CHF +Fab	11	0.2046	0.0494	0.0024
FS9mf	14	0.602	0.0864	0.0075
CHF	14	0.2014	0.0646	0.0042
FS9mf +Fab	16	0.3906	0.1141	0.0130
CHF +Fab	16	0.2488	0.0856	0.0073
FS9mf +unspec.Fab	13	0.6245	0.0751	0.0056
CHF +unspec.Fab	13	0.2077	0.0522	0.0027

Table 1

Invasion index of different cell lines in Abercrombie's confronted explant assay

Invasion index				
cell line	n number of confronted explants	x average of invasion index	s standard deviation of invasion index	s^2 variance of invasion index
3T3	20	0.5090	0.2144	0.0450
CHF	20	0.1635	0.0967	0.0094
3T3t	8	0.7788	0.1272	0.0162
CHF	8	0.2050	0.0477	0.0023
3T3 +Fab	3	0.1767	0.0450	0.0020
CHF +Fab	3	0.1000	0.0000	0.0000
3T3t +Fab	5	0.3360	0.1674	0.0286
CHF +Fab	5	0.1280	0.0649	0.0042
RAT-1	20	0.4425	0.1215	0.0148
CHF	20	0.2305	0.0593	0.0055
RAT-1t	8	0.5070	0.1484	0.0220
CHF	8	0.2030	0.0715	0.0051
RAT-1t +Fab	4	0.2800	0.0878	0.0077
CHF +Fab	4	0.2100	0.0399	0.0012

Table 1 (continued)

Invasion index of different cell lines in Abercrombie's confronted explant system.

A t-test for two independent means between the indices of FS9 cells and FS9_{Fab} cells indicated a test statistic of 8.430 (table 2). Consultation of a t-table for 40 degrees of freedom showed that for the calculated t value of 8.430 the associated error-probability is less than 0.01. The conclusion to be drawn from the confidence level is that there is a highly significant difference in the means between FS9 and FS9+Fab. FS9+Fab cells were strongly contact inhibited, ie. the invasive behavior was strongly inhibited. Chicken heart fibroblasts seemed not to be affected by the Fab treatment. There was no significant modification of invasive behavior of CHF.

A t-test for two independent means between FS9 ↔ FS9 +Fab				
	t	t _{0.05}	t _{0.01}	n+m
FS9 ↔ FS9 +Fab	8.430	2.021	2.705	42
CHF ↔ CHF +Fab	1.863	2.021	2.705	42

Table 2

A t-test of invasion indices for 40 degrees of freedom (n+m-2) for FS9 ↔ FS9 +Fab and for CHF ↔ CHF +Fab from the same confronted explants. The confidence level is 95% and 99%. Numbers in italics indicate that there is a statistically significant difference between the two means. The results show that the effect of Fab on FS9 cell invasivity is very strong.

A test statistic of 1.992 was calculated from a t-test of two independent means between indices of L929 and L929+Fab (table 3). There was no statistical significant difference in the invasive behavior with and without Fab. The Fab did not influence the invasivity of the L929 cells. Contact inhibition was strong with and without Fab. The CHF also failed to show any significant changes in invasivity when Fab was present. In general the CHF had a very low variability expressed in the small standard deviation in table 1. A highly consistent behavior in all the confronted explants was a typical attribute of CHF.

A t-test for two independent means between L929 ↔ L929 +Fab				
	t	t _{0.05}	t _{0.01}	n+m
L929 ↔ L929 +Fab	1.992	2.064	2.797	26
CHF ↔ CHF +Fab	0.455	2.064	2.797	26

Table 3

A t-test of invasion indexes for 24 degrees of freedom (n+m-2) for L929 ↔ L929 +Fab and for CHF ↔ CHF +Fab from the same confronted explants. The confidence levels are 95% and 99%. There is no statistically significant difference in invasion with and without Fab's in these two pairs.

The effect of Fab (0.1 mg/ml) prepared from the anti-p37 monoclonal antibody DD9 on the invasive behavior of p37-free FS9 cells (FS9mf) was monitored. Unexpectedly, the invasion index was reduced, the reduction being significant at a confidence level of 99% (Tab. 4). The contact inhibition of CHF was not affected by Fab treatment.

A t-test for two independent means between FS9mf ↔ FS9mf + Fab				
	t	t _{0.05}	t _{0.01}	n+m
FS9mf ↔ FS9mf + Fab	5.476	2.048	2.763	30
CHF ↔ CHF + Fab	0.314	2.028	2.763	30

Table 4

A t-test of invasion indices for 28 degrees of freedom (n+m-2) for FS9mf ↔ FS9mf + Fab and CHF ↔ CHF + Fab from the same confronted explants. The confidence levels are 95% and 99%. Numbers in *italics* indicate that there is a statistically significant difference between the two means.

The effect of unspecific Fab (0.1 mg/ml) on the invasivity of FS9mf cells was used as control. There was no significant modification of invasion index when unspecific Fab was present (table 5). The CHF also failed to show any significant changes in invasivity.

A t-test for two independent means between FS9mf ↔ FS9mf + unspec.Fab				
	t	t _{0.05}	t _{0.01}	n+m
FS9mf ↔ FS9mf + unspec.Fab	0.720	2.060	2.787	27
CHF ↔ CHF + unspec.Fab	0.267	2.060	2.787	27

Table 5

A t-test of invasion indices for 25 degrees of freedom (n+m-2) for FS9mf ↔ FS9mf + unspec.Fab and for CHF ↔ CHF + unspec.Fab from the same confronted explants. The confidence levels are 95% and 99%. There is no statistically significant difference in invasion with or without unspecific Fab in these two pairs.

The effect on invasivity of inducing p37 on 3T3 cells was of particular interest. The subsequent increase in the invasion index was significant even at a confidence level of 99% (table 6). The 3T3t cells behaved similarly to FS9 cells. It is important to note that CHF did not change their invasion indices in these assays. The possibility existed that p37 was also induced in the CHF cells during the assay. However, induction took at least 7 days (see earlier) and the confronted explant assay lasted only 2 days. Hence, it is unlikely that the CHF cells contained much p37 and the constant invasion indices observed support this possibility.

A t- test for two independent means between 3T3 ↔ 3T3t				
	t	<i>t</i> 0.05	<i>t</i> 0.01	n+m
3T3 ↔ 3T3t	3.210	<i>2.056</i>	<i>2.779</i>	28
CHF ↔ CHF	1.117	2.056	2.779	28

Table 6

A t-test of invasion indices for 26 degrees of freedom (n+m-2) for 3T3 ↔ 3T3t and for CHF ↔ CHF from the same confronted explants. The confidence levels are 95% and 99%. Numbers in italics indicate that there is a statistically significant difference between the independent means. The presence of p37 changes the invasive behavior of the 3T3 cells significantly at confidence levels of 95% and 99%. Only random changes were apparent in CHF behavior.

Unexpectedly, Fab also reduced the invasion index of normal 3T3 cells, although these cells do not possess p37 (table 1). The reduction was significant at a confidence level of 95% (no significance at 99%, table 7. Note: only three assays of 3T3 +Fab are incorporated into the statistical analysis, table 1). No significant changes occurred in the behavior of CHF.

A t-test for two independent means between 3T3 ↔ 3T3 +Fab				
	t	<i>t</i> 0.05	<i>t</i> 0.01	n+m
3T3 ↔ 3T3 +Fab	2.557	<i>2.080</i>	2.831	23
CHF ↔ CHF +Fab	1.227	2.080	2.831	23

Table 7

A t-test of invasion indices for 21 degrees of freedom (n+m-2) for 3T3 ↔ 3T3 +Fab and for CHF ↔ CHF +Fab from the same confronted explants. The confidence levels are 95% and 99% . Numbers in italics indicate that there is a statistically significant difference between the two independent means. The Fab changes the invasive behavior of the 3T3 cells only at a confidence level of 95%. CHF behavior was not significantly affected by Fab.

The effect of Fab (0.1 mg/ml) on the invasive behavior of both 3T3t and 3T3 cells was highly significant (table 7 and table 8). The invasion indices were dramatically reduced. The significance still endured at a confidence level of 99% (table 8). The invasion index of 3T3t cells treated with Fab was lower than that of non-induced 3T3 cells (table 1). The invasive behavior of CHF was again unaffected by the Fab treatment.

A t-test for two independent means between 3T3t ↔ 3T3t +Fab				
	t	<i>t</i> _{0.05}	<i>t</i> _{0.01}	n+m
3T3t ↔ 3T3t +Fab	6.490	<i>2.201</i>	<i>3.106</i>	13
CHF ↔ CHF +Fab	1.422	2.201	3.106	13

Table 8

A t-test of invasion indices for 11 degree of freedom (n+m-2) for 3T3t ↔ 3T3t +Fab and for CHF ↔ CHF +Fab from the same confronted explants. The confidence levels are 95% and 99%. Numbers in italics propose that there is a statistically significant difference between the two independent means. The Fab changes the invasive behavior of the 3T3 cells at a confidence level of 99% and 95%. No significant changes were apparent in the behavior of CHF.

BM-cycline treatment of FS9 cells and the subsequent loss of both, mycoplasmas and p37, had also an effect on invasive behavior. The t-test indicated that FS9 cells without p37 and mycoplasmas (FS9mf) had significantly reduced invasion indices when compared with p37-positive FS9 cells. The chicken heart fibroblasts failed to show any significant changes in invasivity.

A t-test for two independent means between FS9 ↔ FS9mf				
	t	t _{0.05}	t _{0.01}	n+m
FS9 ↔ FS9mf	3.585	2.030	2.7254	38
CHF ↔ CHF	0.253	2.030	2.754	38

Table 9

A t-test of invasion indices for 36 degrees of freedom (n+m-2) for FS9 ↔ FS9mf and for CHF ↔ CHF from the same confronted explants. The confidence levels are 95% and 99%. Numbers in *italics* indicate that there is a statistically significant difference between the independent means.

RAT-1 cells contained p37 and we wished to determine whether treatment with FS9 cell culture supernatant lead to any changes in invasive behavior. However, no significant differences in the invasive indices of RAT-1 and RAT-1t cells were detected (table 10). CHF behavior was not affected.

A t-test for two independent means between RAT-1 ↔ RAT-1t				
	t	t _{0.05}	t _{0.01}	n+m
RAT-1 ↔ RAT-1t	1.044	2.056	2.779	28
CHF ↔ CHF	0.982	2.056	2.779	28

Table 10

A t-test for invasion indices for 26 degrees of freedom (n+m-2) for RAT-1 ↔ RAT-1t and for CHF ↔ CHF from the same confronted explants. The confidence levels are 95% and 99%. Within these confidence levels there is no statistically significant difference in invasive behavior between RAT-1 and RAT-1t as well as between CHF and CHF.

We examined the effect of Fab (0.1 mg/ml) on the invasive behavior of RAT-1t cells. The drastic reduction in the invasion index when Fab was present was significant at a confidence level of 95%. (Note: only four assays of RAT-1t +Fab are incorporated into the statistical analysis). The behavior of CHF was as usual unaffected by Fab.

A t-test for two independent means between RAT-1t ↔ RAT-1t +Fab				
	t	<i>t</i> 0.05	<i>t</i> 0.01	n+m
RAT-1t ↔ RAT-1t +Fab	2.468	2.228	3.169	12
CHF ↔ CHF +Fab	0.160	2.228	3.169	12

Table 11

A t-test of invasion indices for 10 degrees of freedom (n+m-2) for RAT-1t ↔ RAT-1t+Fab and for CHF ↔ CHF +Fab from the same confronted explants. The confidence levels are 95% and 99%. Numbers in italics indicate that there is a statistically significant difference between the independent means. The Fab changed the invasive behavior of RAT-1 cells significantly at a confidence level of 95%. No significant changes were apparent in the behavior of CHF.

6. Time Lapse Film Analysis of Cell Collisions Shows that P37 Influences Contact Inhibition of Locomotion

In an attempt to insure and verify our results we decided to film single cell collisions as a different method to analyse cell behavior.

Collisions of single FS9 cells and single chicken heart fibroblast cells were filmed. The path of the nucleus, drawn as a series of sequential points representing 1 min intervals, was fed into a PDP 11/44 computer and changes in motion were analysed by a program developed by Dr A. Brown and Dr G. Dunn (Paddock and Dunn, 1986). The calculated x-means represent the scalar difference between the distance a cell has actually progressed during the 10 minutes following the first contact in the direction it was moving for the 10 minutes prior to the collision and the distance it would have progressed had there been no change in motion. The sampled values shown in table 12 represent the horizontal components of scaled accelerations. A selection for velocity larger than $0.5\mu\text{m min}^{-1}$ was made. The more negative the x-means, the greater the inhibition of locomotion. The data on changes in motion were summarized diagrammatically in a so-called accelogram (Fig. 23). Each accelogram was constructed from a sample of accelerations, all obtained using the same time interval of 10 minutes. The samples were gathered together so that they had a common origin at the center of a circle of radius 1. The accelograms show the changes in motion in relation to the initial direction of motion. Each acceleration was oriented so that the displacement prior to the collision would be horizontally on the center line. For example, a hypothetical cell would have a vector A in Fig. 22. This indicates a change of direction (following the collision) by 60° to the left and an increased speed by a factor of 1.5. Vector B would represent a change in direction of 90° to the right and a decreased speed by a factor of 0.5 (Fig. 22).

Normal p37-positive FS9 cells had a x-mean of -0.26110. They represent the least contact inhibited FS9 cells. When Fab (0.1 mg/ml) directed against p37 was present these cells showed a strongly altered contact behavior. The x-mean (-0.77229) indicated a significantly higher contact inhibition as a result of Fab treatment.

The mean calculated from BM-cycline treated (p37 negative and mycoplasma free) FS9 cells was -0.37268, which is a value between normal p37-positive and Fab-treated FS9 cells. Once again the p37-negative FS9 cells were even more contact inhibited when Fab against p37 was present.

Chicken heart fibroblasts were all less contact inhibited than expected (table 12). The reasons for this phenomenon will be discussed later (see Discussion).

Contact acceleration index (without subtraction of x-means of freely moving cells)					
cell line	Number of analysed cells (n)	contact acceleration index	cell line	Number of analysed cells (n)	contact acceleration index
FS9	10	-0.26110 $\pm$ 0.10890	CHF	8	-0.36619 $\pm$ 0.10951
FS9+Fab	12	-0.77292 $\pm$ 0.06106	CHF	2	-0.34607 $\pm$ 0.11339
FS9mf	20	-0.37286 $\pm$ 0.05987	CHF	14	-0.27991 $\pm$ 0.10670
FS9mf+Fab	11	-0.45521 $\pm$ 0.15268	CHF	6	0.01079 $\pm$ 0.18076

Table 12

Contact acceleration indices of single cell collisions between either FS9 (with and without DD9 Fab) or FS9mf (with and without DD9 Fab) and chicken heart fibroblasts. A selection for velocity larger than $0.5 \mu\text{m min}^{-1}$ was made. The means are without subtraction of scaled values of freely moving cells. (mf = mycoplasmafree)

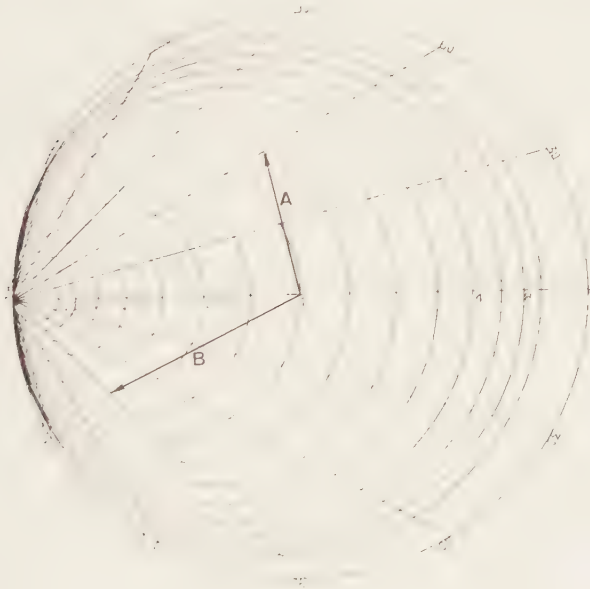


Fig 22

Accellogram of 2 hypothetical cells. See text for further details.

Fig 23

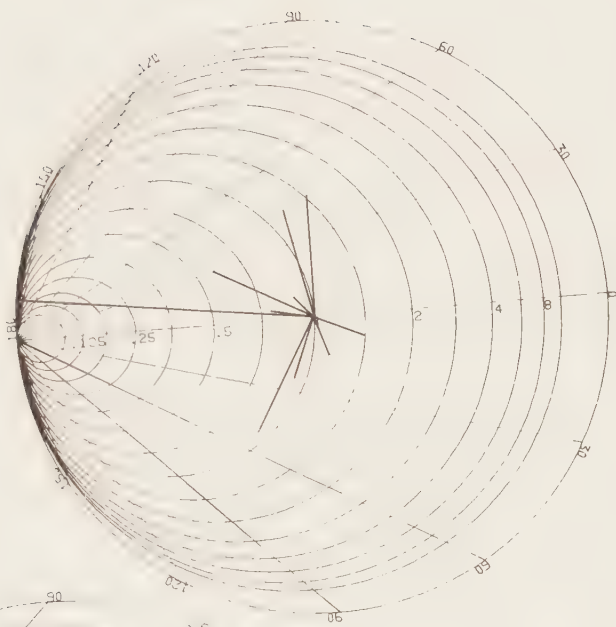
Accelogram of either FS9 cells or chicken heart fibroblasts during collisions obtained from time lapse film-analysis. Each acceleration is represented by a straight line with its origin at the center of the diagram. The time of the analysis was ten minutes before and after the first contact between FS9 cells and chicken heart fibroblasts.

(a) Accelogram of 8 FS9 cells colliding with chicken heart fibroblasts.

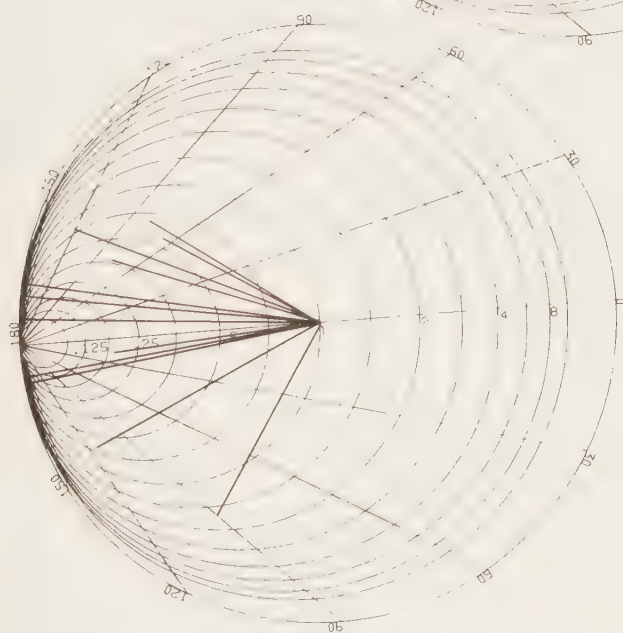
(b) Accelogram of 12 FS9 cells colliding with chicken heart fibroblasts in the presence of 0.1 mg/ml Fab (DD9).

(c) Accelogram of 20 mycoplasma-free and p37-negative FS9 cells colliding with chicken heart fibroblasts.

(d) Accelogram of 11 mycoplasma-free and p37-negative FS9 cells colliding with chicken heart fibroblasts in the presence of 0.1 mg/ml Fab (DD9).

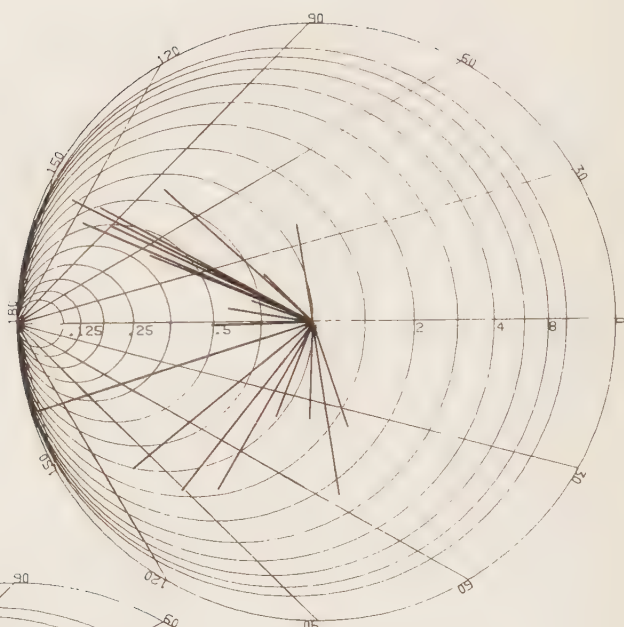


(a) $n=10$

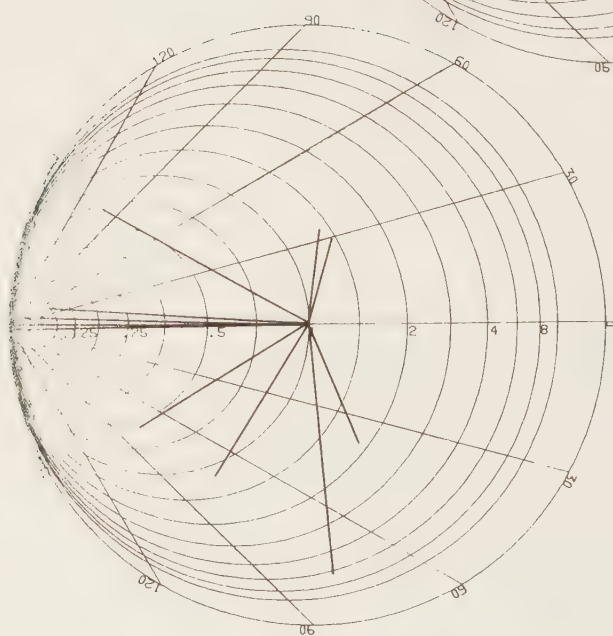


(b) $n=12$

Fig 23 (a), (b) continued



(c) $n=20$



(d) $n=11$

Fig 23 (c), (d) continued

7. P37 is Constituent of Mycoplasma Hyorhinis

The experiments with BM- cycline indicated that p37 was a mycoplasma protein and we wished to identify the Mycoplasma species which it was associated with.

Consequently, we attempted to isolate and identify the mycoplasma species in our cell cultures. This work was done in collaboration with Dr. R. H. Leach, Mycoplasma Reference Facility, NCTC London. Two species were initially identified, Mycoplasma arginini and Mycoplasma orale (Table 13). The presence of Mycoplasma arginini in FS9 and A375 M cells, both positive for p37, combined with its absence from L929 and A375 P cells, suggested it may be the source of p37. However, Mycoplasma arginini isolated from FS9 cells and grown *in vitro* did not contain p37 (Fig. 24a). Furthermore, when mycoplasma-free L929 cell cultures were inoculated with either Mycoplasma arginini or Mycoplasma orale, p37 did not appear. Hence, the two mycoplasma were not inducing the synthesis of a cellular protein. DAPI-staining indicated mycoplasma infection had occurred. The p37-positive human melanoma cell line, MM96, was contaminated with Mycoplasma hyorhinis. However, this particular strain could not be cultivated *in vitro*. Consequently, we used Mycoplasma hyorhinis GDL, a strain which grows *in vitro*. Western blotting indicated the presence of p37 in Mycoplasma hyorhinis GDL (Fig. 25a). When L929 cells, either mycoplasma-free or contaminated with Mycoplasma orale, were inoculated with Mycoplasma hyorhinis GDL, western blotting showed they had become p37 positive after 7 days in culture (Fig. 25b).

Mycoplasma isolation and identification			
Cell lines	correlation with p37	DAPI stain	Mycoplasmas identified
FS9	p37 +	positive	M.arginini
L929	p37 -	positive	M.orale
A375 M	p37 +	positive	M.arginini
A375 P	p37 -	positive	M.orale
PC-3P	p37 +	positive	not identified
PC-3M	p37 +	positive	not identified
MM96	p37 +	positive	M.hyorhinis
B16 F1	p37 -	negative	---
B16 F10	p37 -	positive	not identified
FS9 + BM-cycline	p37 -	negative	---

Table. 13

Identification of mycoplasma species contaminating different cell lines and their correlation with p37.

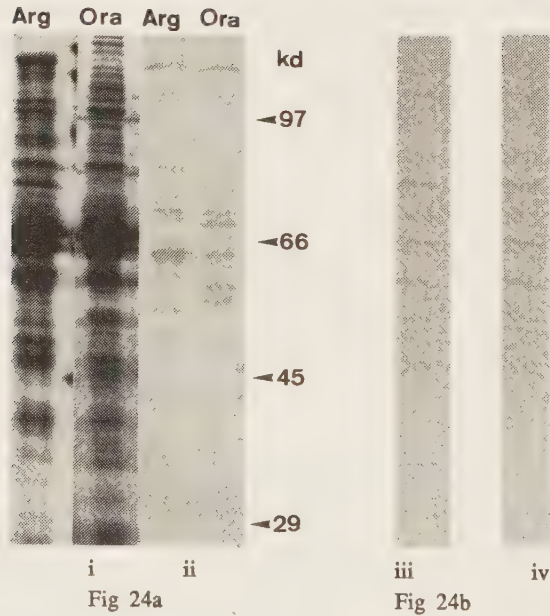


Fig. 24

Assays for the presence of p37 in *Mycoplasma arginini* and *Mycoplasma orale* and the ability of the two mycoplasmas to induce p37 in L929 cells.

- a) Lysates of *arginini* and *orale* were separated with 12% SDS PAGE. The gels were either stained with coomassie blue (i) or transferred electrophoretically to a nitrocellulose filter (ii). Filters were incubated with the anti-p37 antibody DD9. No p37 was detectable.
- b) L929 cell cultures (mycoplasma-free) were infected with *Mycoplasma arginini* (iii) or *Mycoplasma orale* (iv). The crude membranes were isolated, run on 12% SDS PAGE, electroblotted and the filters incubated with DD9. No p37 was detectable.

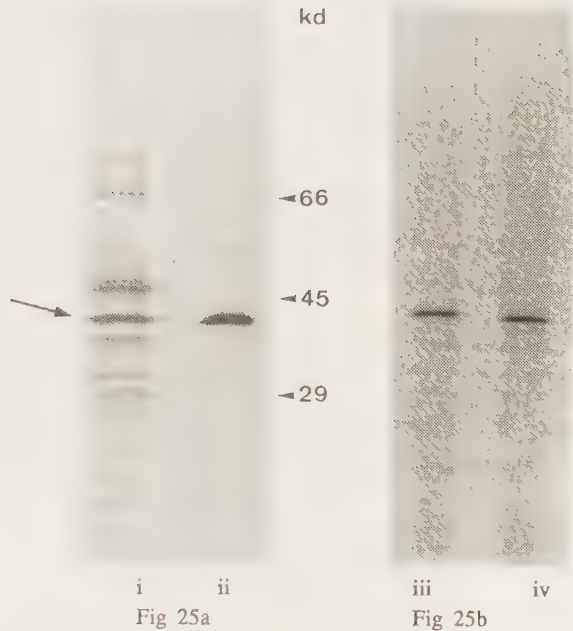


Fig. 25

Assays for the presence of p37 in *Mycoplasma hyorhinis* and the ability of *Mycoplasma hyorhinis* to "induce" p37 on L929 cells.

a) Proteins of *Mycoplasma hyorhinis* were separated with 12% SDS PAGE. The gel was either stained with coomassie blue (i) or western blotted using the monoclonal antibody DD9 to detect p37 (ii). P37 is a component of *Mycoplasma hyorhinis* and corresponds to one of the major coomassie blue stained bands (arrow).

b) Proteins present in L929 cells, contaminated with *Mycoplasma orale* (iii) or mycoplasma-free L929 cells (BM-cycline treated) (iv) which had been infected with *Mycoplasma hyorhinis*. The crude membranes were separated with a 12% SDS PAGE. The presence of p37 was monitored using the DD9 monoclonal antibody.

8. Mycoplasma Hyorhinis is Located at the Cell Surface and is not Released by P37-specific Antibody

The discovery that p37 is associated with Mycoplasma hyorhinis and is present in cell cultures as a result of infection by that organism raised a number of questions regarding the effects of anti-p37 Fab in the confronted explant test. The Fab might, for example, cause the release of mycoplasma from the cell surface. FS9 cells were incubated with 0.1 mg/ml Fab (DD9) overnight at 37°C (the same conditions as in the confronted explant system). Next day the cells were fixed and the mycoplasma monitored by fluorescence microscopy using a FITC-labelled rabbit anti-mouse antibody. Intensive fluorescence could be detected uniformly distributed on the cell surface indicating that the mycoplasmas were not released from the fibroblasts during the Fab treatment (Fig. 26).

Following incubation of FS9 cells with the DD9 antibody carried out under conditions identical to those described above, a typical capping of the antigens (ie. mycoplasma) was observed (Fig. 27).

To ascertain that Mycoplasma hyorhinis was being labeled by the anti-p37 DD9 antibody, triple labeling was carried out using DD9, DAPI and a hyperimmune specific rabbit sera prepared against Mycoplasma hyorhinis (a gift from Dr. R. H. Leach). The mouse antibody DD9 was visualised with a FITC-labeled rabbit anti-mouse antibody and the rabbit antibody was visualized with a TRITC-labeled goat anti-rabbit antibody. The FITC, TRITC and DAPI staining was coincident (Fig. 28).



Fig. 26
Effect of DD9 Fab on the association of *Mycoplasma hyorhinis* with FS9 cells.

Living FS9 cells were incubated overnight with 0.1 mg/ml Fab. Indirect immunofluorescence was carried out using a FITC labelled rabbit anti-mouse antibody (a), the same cell stained with the DNA stain DAPI (b). The treated cells did not differ from untreated controls (not shown).

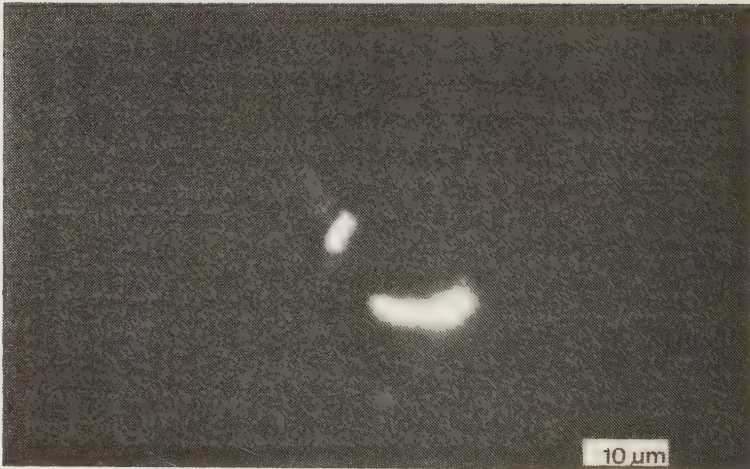


Fig. 27

Capping of p37 on FS9 cells treated with DD9 IgG.

Cells were incubated with IgG overnight and antigen visualized using FITC-labelled rabbit anti-mouse antibody.

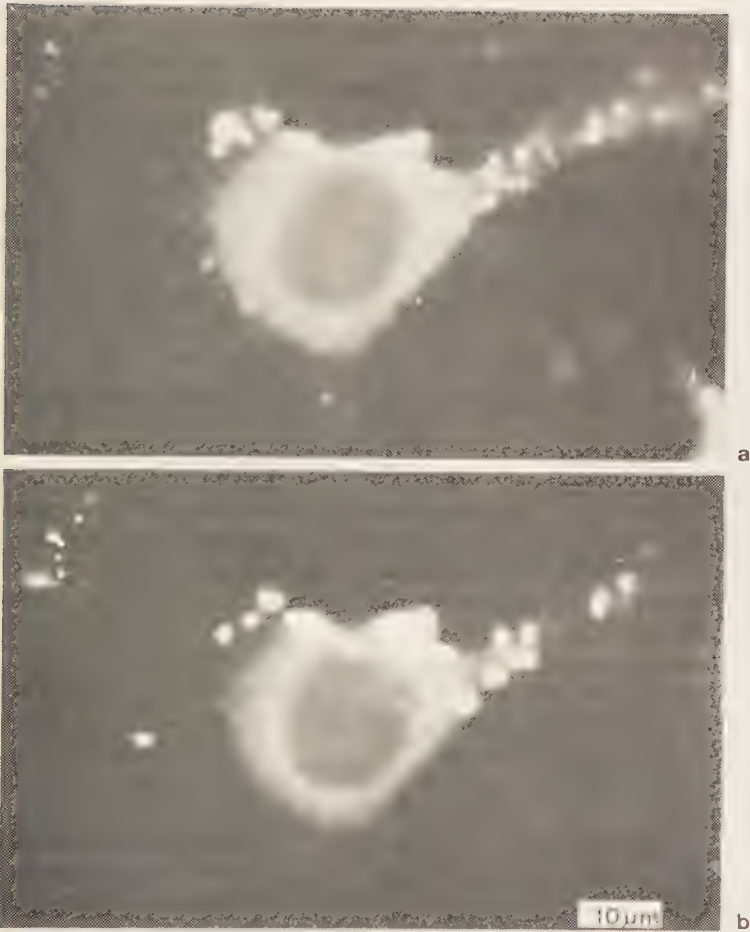


Fig. 28

Evidence that p37 is associated with *M. hyorhinis* in FS9 cell cultures.

Cells were labelled with a hyperimmune specific rabbit sera against *M. hyorhinis* (a), anti-p37 DD9 antibody (b) and the DNA stain DAPI (c). The same cells in phase contrast are shown in (d).



Fig. 28
(continued)

DISCUSSION

Screening for P37

Immunoblotting with the monoclonal antibody DD9 was used to screen a variety of metastatic and non-metastatic cell lines for the presence of p37. P37 was found associated with the FS9 mouse sarcoma cells, the highly metastatic human melanoma A375 M, the malignant human melanoma MM96, the malignant human prostate adenocarcinoma PC-3 M as well as with its non-malignant human parent line PC-3 P. RAT-1 fibroblasts cells also contained p37.

We failed to detect any p37 in the non-invasive mouse sarcoma cell line L929, the low metastatic human melanoma cell line A375-P or in NIH 3T3 mouse fibroblasts. Neither the non-metastatic mouse melanoma B16 F1 nor its highly metastatic variant B16 F10 carried the antigen.

These results showed that metastatic / invasive behavior was not invariably correlated with the presence of p37. The association of p37 with both the metastatic and the non-metastatic variants of the human prostate carcinoma imply the antigen is not alone sufficient to elicit metastatic behavior. (However, we have not compared the invasivity of these two lines in the confronted explant assay.)

The binding of the DD9 monoclonal antibody to cells was quantitated using a protein A binding assay. Embryonal chicken heart fibroblasts gave, as expected, a very low signal which presumably represented background as it remained constant in all the dilution steps. The signal was equivalent to that obtained when FS9 cells were incubated with non-specific rabbit anti-mouse antibody instead of DD9.

Although formaldehyde fixed L929 cells gave negative results when DD9 binding was examined using indirect immunofluorescence and no p37 was identified with immunoblotting, they did give a positive, albeit weak signal in the protein A assay. The binding showed a prozone effect. Similarly, the A375-P cells, which also gave negative results in immunoblots and immunofluorescence studies, were weakly positive in the protein A assay. Consequently, L929 and A375-P cells may carry an epitope which is shared by p37. Whether this second epitope is also present on p37-positive cells is not known.

Confronted Explant Assays

When cells come into contact with each other, their behavior is often considerably altered. An example of this is the phenomenon of contact inhibition of locomotion, first described

in detail by Abercrombie and Heaysman (1954). Abercrombie (1970) and Harris (1974) reported two main types of contact inhibition. In the first type, a cell probably stops upon collision because it is more firmly attached to the substrate than to the cell with which it collides. The second type involves direct inhibition of the cellular machinery of locomotion. This inhibition may result from signals, triggered by direct contact of the cell surfaces, which alter cellular behavior.

Normally, cells exhibit homotypic contact inhibition. This reflects the force of the outward radial migration of cells from an explant. Projan and Tannberger (1973) showed human malignant cells possess much greater variation in homotypic contact inhibition than normal fibroblasts. Gulstein et al., (1973) found no deficiency in homotypic contact inhibition between transformed and normal cells, while Vasley and Weiss (1973) showed that neoplastic rat fibroblast cells have a high degree of homotypic contact inhibition. Whether homotypic contact inhibition is related to the invasiveness of the cells is not clear. It is not known whether there is any correlation between homotypic and heterotypic contact inhibition or if there are two different independent contact inhibition systems.

Tumor cells escape from normal contact inhibition. Malignant tumors often occupy territory held by normal fibroblasts but not *vice versa*. This infiltration and overgrowth is called non-reciprocal invasion (Abercrombie et al., 1957; Stephenson et al., 1974; Abercrombie and Heaysman, 1976; Heaysman and Turin, 1976; Heaysman, 1978; Stephenson and Stephenson, 1978; Abercrombie, 1979; Stephenson, 1982a; Stephenson, 1982b; Steinemann et al., 1984; Paddock and Dunn, 1986). Abercrombie (1975) defined cellular invasion as the movement of a cell population into an area already occupied by another population. There is considerable evidence that the behavior of two cell types during and following collision mimics aspects of certain biological processes, including malignant invasion (Abercrombie, 1979; Parish et al., 1987).

We used the confronted explant assay for assessing cellular invasion. In this method two foci of cells (or explants) are placed about 1mm apart on a cover slip in medium and the behavior of the cells observed when the outwandering populations meet. Invasion indices were computed from fixed explant assays. Steinemann et al., (1984a,b) studied the effects of Fab directed against p37, a surface protein of chemically-induced fibrosarcoma cells (FS9), on the non-reciprocal invasion of CHF by FS9 cells. We have used the same system and have calculated an invasion index of 0.7725 for FS9 cells, a ratio derived from 24 explants. Monoclonal Fab (0.1 mg/ml) directed against p37 reduced the invasive behavior dramatically. The invasion index of the non-invasive control cell line L929 did not change significantly after treatment with Fab. When Fab derived from control IgG or from anti-L929 plasma membrane rabbit-antiserum preabsorbed with FS9 cells was used, no effect on FS9 cell invasivity was observed (Steinemann et al., 1984a,b).

Since p37 disappeared from BM-cycline treated cells we were able to determine any resulting changes in invasive behavior. There was, as expected, a significant reduction in the invasion index although the cells were still invasive. Unexpectedly, the addition of specific Fab (0.1 mg/ml) directed against p37 to p37-free FS9 cells significantly reduced

the invasion index further. We also examined the effect of unspecific Fab on the invasive behavior of p37-free FS9 cells. No significant modifications of the invasion index were detected.

These results suggest that the reduction of invasiveness following addition of monoclonal Fab against p37 was not an unspecific Fab-effect. On the other hand, the mechanism by which the Fab inhibits the invasivity of p37-free FS9 cells is not clear. Immunofluorescence and immunoblotting have so far failed to detect additional epitope(s) on the latter cells that may bind the antibody. The fact that some p37-free cells give positive results in the protein A assay indicates such additional epitope(s) may be present. Fixation with formaldehyde and solubilisation with Triton X-100 apparently denature or block the epitope.

There is a correlation between the occurrence of p37 and invasive behavior *in vitro*. The presence of p37-specific Fab reduced the invasive index of FS9 cells more than the removal of p37 by BM-cycline treatment. Whether this is related to a second unidentified epitope on the cell surface is not known.

The invasivity of p37-free NIH-3T3 mouse fibroblasts was also assayed in Abercrombie's confronted explant test. The invasion index of these cells lies between the indices of FS9 and L929 cells. However, there is considerable variation in the invasion indices observed in different NIH 3T3 cell explants. The metastatic behavior of these cells *in vivo* is also variable. Greig et al., (1985) transfected NIH 3T3 cells with the c-Ha-ras 1 gene and found that, in the mouse, both tumorigenicity was accelerated and metastatic potential enhanced. However, under specific conditions (eg. inoculation in the food pad) non-transfected cells were also metastatic *in vivo*.

Since it was possible to transmit p37 to 3T3 cells (see later), we compared invasion indices of p37-free 3T3 and 3T3t (i.e. carrying p37) fibroblasts and the effect of Fab directed against p37. The results suggested that, if p37 was present, the fibroblasts became more invasive. Moreover, if p37 was blocked with specific Fab, the invasive behavior was once again reduced. There was a further reduction of invasivity when p37-free 3T3 cells were treated with Fab directed against p37 below the level of untreated normal 3T3 cells.

RAT-1 fibroblasts possessed p37. As anticipated, incubation of these cells with FS9 cell culture supernatant did not result in an increase in p37 and their invasive behavior was not changed. Once again a dramatic reduction in the invasion index resulted from adding 0.1 mg/ml Fab to the confronted explant assay. Assays with BM-cycline treated RAT-1 cells were not carried out.

The studies showed that cells carrying p37 were the most strongly invasive in the confronted explant test. The removal of p37 by treatment with BM-cycline consistently resulted in reduction of invasivity. Treatment with Fab specific for p37 caused loss in invasiveness of both p37-positive and p37-free cells when they had been invasive before the treatment.

Time Lapse Film Analysis

Paddock and Dunn (1986) have studied contact inhibition of locomotion by analysing the changes in cellular locomotion resulting from single cell collisions. In particular, they have examined the heterotypic collision between a single FS9 cell and a chicken heart fibroblast in the absence of simultaneous contact with several neighbouring cells (i.e. homotypic contact inhibition). They found that there is an active invasive response of the FS9 cell (contact promotion) following collision with a chicken heart fibroblast. This was surprising as FS9 populations were slightly contact inhibited in confronted explant assays (Abercrombie, 1979; Steinemann et al., 1984ab). Paddock and Dunn (1986) concluded that the behavior of the FS9 cells cannot simply be explained by a suppression or failure of contact inhibition since these cells showed a tendency to turn towards the marginal collision point.

Our FS9 cells did not show contact promotion in single cell collisions with chicken heart fibroblasts, although contact inhibition was minimal. This difference may reflect slight differences in the cell lines. We used cell lines grown in culture whereas Paddock and Dunn employed cells taken directly from tumors in mice. Our FS9 cells have lost the ability to form tumors when injected into mice. They also differ morphologically, spread less and were more rounded than the cells used by Paddock and Dunn (Dunn, personal communication). Unfortunately, the original cell line has been lost, so we were unable to compare it with our FS9 cells.

The results obtained with time lapse filming of single cell collisions appeared to be comparable with those obtained from confronted explant studies. P37-positive FS9 cells were the most invasive population and showed the largest failure of contact inhibition following the single cell collisions. The presence of Fab directed against p37 resulted in strong contact inhibition of FS9 cells. Furthermore, film analysis indicated BM-cycline treated FS9 cells (p37-negative) were more contact inhibited than their p37-positive counterpart. Anti-p37 Fab also decreased the heterotypic contact inhibition of p37-negative FS9 cells. But this effect was not as strong as the effect of Fab to p37-positive cells.

The behavior of the chicken heart fibroblasts in the single cell collisions was unexpected. Contact inhibition was low following collision with FS9 cells, although the confronted explant studies indicated invasion of the FS9 cell population was in fact minimal. Paddock and Dunn (1986) found the chicken heart fibroblasts were strongly contact inhibited following collisions with FS9 cells. The single cell collision are studied in sparse cultures whereas cell density is high in the confronted explant assay. These are fundamentally different systems. Our FS9 cells may release a factor stimulating the movement of chicken heart fibroblasts. The single chicken heart fibroblasts may react differently when they are out of contact with any neighbor cells. Another hypothesis would be that just single FS9 cells, without neighbouring contact such as in confronted explant tests, are able to release a stimulating factor.

As is known from the literature, several factors released from cells may alter the cell

behavior. For example, platelet-derived growth factor (PDGF) acts as a chemoattractant stimulating the migration of fibroblasts (Grotendorst, 1984; Heath and Ireland, 1985; Seppä et al., 1982). Angiogenic factors induce increased motility of endothelial cells as well as higher expression of plasminogen activators (Gross et al., 1983). Liotta et al., (1986) characterized an autocrine motility factor in tumor cells. Stocker (1973) proposed that emigrating cells at the wound edge travel through and respond to a concentration gradient of an unknown factor in the surrounding medium.

A more likely explanation for the lack of chicken heart fibroblast contact inhibition may be that we did not limit our analysis to the head to head collisions between FS9 cells and chicken heart fibroblasts. Boyde et al., (1969) found that, when the collision is between the leading lamella of one fibroblast and the side of another, the forward movement of the first fibroblast may continue for a very short time but then usually contact inhibition becomes effective. However, the movement of the second fibroblast is unaffected.

(Mycoplasmas were not involved in the failure of chicken heart fibroblasts contact inhibition in single cell collisions since in mycoplasma-free assays the same effect was also observed).

The Source of P37

After discovering that a p37-free cell line acquired p37 when incubated with sterile filtered culture supernatant of cell lines containing p37, we contemplated that either infectious agents, such as viruses and mycoplasmas induce or carry the antigen, or that it is induced by factors such as autocrine regulators.

Liotta et al., (1986) characterized an 'autocrine motility factor' AMF which can stimulate the motility of tumor cells. Host serum factors or connective tissue breakdown products can be chemotactic for certain types of tumor cells (Lamm et al., 1981; McCarthy et al., 1985). Since unrestrained tumor growth is thought, in part, to be caused by autocrine growth factors, it appeared reasonable that other aspects of invasive behavior might also be regulated by autocrine mechanisms (Todaro et al., 1980; Azano et al., 1983; Albin et al., 1986; Dickson et al., 1987). However, the amino acid sequence of p37, which has recently been obtained, does not show sequence homology to any known growth factors (see later).

The relationship between retroviruses, tumors and oncogenes has been extensively reviewed (Bishop 1982, Weinberg 1983). Recent studies showed that primary and established rodent fibroblasts, following transformation by activated *ras* sequences, can form metastases

(Greig et al., 1985; Liotta, 1986; Muschel et al., 1985; Stackpole et al., 1985). Egean et al., (1987) proposed that *ras* is directly involved in regulation of the metastatic phenotype through expression and action of its gene product.

Retroviruses could be isolated from FS9 cells. However, they did not carry p37 and failed to induce the antigen when added to p37-free cell lines (Meier, 1987). Hence, retroviruses are unlikely to be involved in the 'induction' of p37 in cells incubated with FS9 culture supernatant.

Mycoplasma contamination of cultered cells was first reported by Robinson et al., (1956). Subsequent developments have been reviewed by Stanbridge (1971). Although mycoplasmas are common contaminants of tissue culture many investigators have ignored their presence in experimental procedures. Mycoplasmas reside predominantly in the extracellular environment in close association with the host plasma membrane (Powell et al., 1976; Leith and Baseman, 1984; Loomes et al., 1984). When examined by electron microscopy they occasionally appear to be free in the cytoplasm of degenerating cells or within cytoplasmic vacuoles. Stanbridge and Doerson (1981) described modulation of host cell plasma membranes, changes in the tumorigenic potential of malignant cells and alterations of host enzyme patterns as effects that mycoplasma might have upon cells in culture. Kluve et al., (1981) noted during studies on collagenase activity in Balb/c 3T3 cells that cultures contaminated with mycoplasmas had greatly increased enzyme activity.

Many parasites and cancer cells secrete or express on their cell surface degradatory enzymes that presumably facilitate their invasion of host tissues (Pino-Heiss et al., 1985).

The neuramidase of *Mycoplasma gallisepticum* may attack the sialo-glycoproteins on the host cell surface, modifying the cell charge and consequently affecting contact inhibition and cell adhesiveness (Sethi and Müller, 1972).

The presence of mycoplasma infection in our cell cultures was determined using the DAPI DNA-staining technique. Most of the cell lines we studied were positive for mycoplasma, irrespective of their invasive behavior. *Mycoplasma hyorhinis* that was isolated and identified from the p37-positive human melanoma cell line MM96 could not be cultivated *in vitro*. Consequently, we used a *Mycoplasma hyorhinis* strain (*Mycoplasma hyorhinis* GDL) that grows *in vitro*. Immunoblots of proteins isolated from this strain were positive for p37 and L929 cells infected with *Mycoplasma hyorhinis* GDL subsequently became p37-positive.

Leith and Baseman (1984), Loomes et al., (1984) and Powell et al., (1976) described specific attachment of *Mycoplasma pneumoniae* to target cells by a 165 kilodalton surface protein, designated P1. We speculated that p37 may be involved in the attachment of *Mycoplasma hyorhinis* to cells. However, the p37-specific monoclonal antibody failed to release the mycoplasmas from the cell surface. Although p37 may be involved in attachment via another epitope, the experiment does indicate that the antibody does not modify the invasive behavior of cells by releasing mycoplasma from their surfaces.

The hypothesis that adherence leads to fusion of mycoplasma cell membranes with those

of its host has been proposed by Gabridge et al., (1977). If fusion does occur, then a wide variety of proteins (enzymes) and lipids can be introduced directly into the host cell and probably change its behavior. Poste and Nicolson (1980) found that fusion of membrane vesicles from highly metastatic B16 F10 melanoma cells with poorly metastatic B16 F1 cells increased the ability of the F1 cells to become arrested in the lung and form metastases there. Whether p37 is the crucial component in Mycoplasma hyorhinitis fusion could, for example, be tested by incorporating the purified protein into liposomes and fusing them with cells. The effects on invasive behavior could then be monitored.

A non-specific mitogenic effect of Mycoplasma pulmonis and Mycoplasma pneumoniae on lymphocytes has been reported (Biberfeld and Gronowicz, 1977). The chemical nature of the mitogen is unknown. However, inhibition of mitosis of lymphocytes *in vitro* by mycoplasmas has also been described (Callewaert et al., 1975).

Kahane and Brunner (1977) and Goel and Lemke (1975) have reported that a membrane protein isolated from membranes of Mycoplasma pneumoniae and Mycoplasma gallisepticum is glycosylated. However, Archer and Rockwell (1982) found no evidence for the presence of glycoproteins in Mycoplasma gallisepticum. Steinemann et al., (1984a), using a modified silver stain and sodium borohydride-reduction of oxidized carbohydrates, obtained evidence that p37 is glycosylated.

However, we found no evidence for p37 to be a glycoprotein, using glycoprotein specific silverstaining methods and periodic acid Schiff's staining (PAS) as well as binding studies with different lectines (R. Dudler, Th. Schmidt, personal communication)

Mechanisms which may Modify Cell Behavior

The ability of metastasizing tumors to penetrate extracellular matrixes such as the basal membrane has been correlated with an increased production of proteolytic enzymes (Mullins and Rohrlight, 1983). These include plasminogen activator (Dano et al., 1985), lysosomal hydrolases (Sloane and Honn, 1984) and collagenases (Liotta et al., 1982). Specific inhibitors of proteases block tumor invasion *in vitro* and *in vivo* (Rossman and Troll, 1980; Mignatti et al, 1986; Martisian et al., 1986). The release of proteases may well contribute to the ease of penetration: degradation of extracellular matrix components may remove a mechanical constraint on infiltration by the tumor cells. Modification of the surface properties by proteases may contribute to altered behavioral responses. On the other hand, some invasive cells, such as lymphocytes, produce almost no proteases whereas tissue macrophages, with large quantities of hydrolytic enzymes, tend to be non-invasive. Since it is known that mycoplasmas express degradatory enzymes on their surfaces, one could argue that these are responsible for the increased invasive behavior of

cells infected with *Mycoplasma hyorhinis*. However, this would imply that other mycoplasma species, such as *Mycoplasma arginini* and *Mycoplasma orale*, release lower amounts of hydrolases or hydrolases that are less efficient in degrading the extracellular matrix.

In theory, cells with a relatively lower adhesion to the underlying substrate will invade a population of cells which adhere more strongly. *Mycoplasma hyorhinis* might change the substrate adhesivity of cells.

Intercellular interactions are believed to play cardinal roles in embryogenesis, histogenesis and metastasis. On the molecular level, cell-cell adhesion has been shown to depend on the presence of specialized contact receptors known as cell adhesions molecules or CAMs (for reviews see Takeichi et al., 1985; Obrink, 1986). Heterotypic adhesion between liver cells and lens cells has recently been shown to involve L-CAM and N-CAM, respectively (Volk et al., 1987). Greenberg et al., (1984) showed that transformation of rat cerebellar cells with Rous sarcoma virus resulted in decreased N-CAM expression coupled with a decrease in cell-cell adhesion. These adhesion areas may be regions of intercellular communication. Fentiman and Taylor-Papadimitriou (1977) suggested that malignant change might be associated with both a loss of ability of cells to communicate and a loss of specificity in communication. They suggest these changes could be selectively advantageous for metastasis of cancer cells enabling them, as non-communicators to ignore inhibitory signals or, as non-selective communicators to receive stimulatory messages from abnormal sources such as the surrounding cells. Transformed cells which lack contact inhibition form just infrequently gap-junctions between each others. In contrast, normal fibroblasts, surrounded by others, cease movement and make gap-junctions at a high frequency.

The hypothesis would be that signals pass between the cells; signals which might be constituted of diffusible chemicals or of changes in membrane potential for example.

Flaxman et al., (1969) studied the tight junctions which might further contact inhibition by allowing the passage from cell to cell of small ions which can alter cytoplasmic motility. Fine and Taylor (1976) suggested the change in the polymerizability or membrane association of actin, which accounts for a strongly altered lipid bilayer occurring after SV40 transformation, may result in the membrane related changes such as in contact inhibition of locomotion. If, upon the stimulus of contact, adhesive molecules move within the fluid bilayer to the site of contact, such a change as described by Fine and Taylor might prevent the appearance of such a contact specialisation.

The idea that exchange of a signal through specialized contacts causes inhibition of locomotion is an attractive one. We know that the p37 gene is part of an operon which contains a transport ATPase (see later). This suggests p37 is a constituent of a permease complex and hence may be capable of changing the local concentrations of chemical signals.

Chemotactic factors released by cells may also influence invasive behavior, although there

exists no direct evidence for such a mechanism. *Mycoplasma hyorhinis* could modify the release or breakdown of such factors, perhaps even itself release chemotactic factors.

As already discussed, the inhibition of *in vitro* invasion by p37-specific Fab was observed with cells lacking as well as those carrying the antigen. The most obvious explanation is that the former cells possess an epitope resembling that recognised by the antibody on the mycoplasma protein. This could mean both antigens, cellular and mycoplasmal, have similar properties and functions. However, until the cellular antigen is characterized, we cannot exclude the possibility that the Fab is blocking two independent mechanisms of invasion.

Permease Operon

A clone containing the gene coding for p37 has now been isolated and sequenced (Dudler et al., in preparation). The p37 gene appears to be the first in an operon coding for three proteins. The amino acid sequence of the second protein, deduced from the DNA sequence, is homologous to a class of procaryotic and eucaryotic transport ATPases. The DNA sequence indicates that p37 is synthesized as a precursor with a typical leader sequence. This is consistent with p37 being a membrane protein exposed on the mycoplasma surface.

Speculations about the Mode of Action of P37

The mechanisms of contact inhibition and invasiveness are poorly understood. It is not known whether homotypic and heterotypic contact inhibition of locomotion are regulated by two independent systems. Since, in the animal, malignant cells invade populations of normal cells it is probable that their heterologous contact inhibition with normal cells has more relevance to their invasive capacity.

We have not looked at homotypic contact inhibition in our studies on single cell collisions. However, the confronted explant assays did not indicate the anti-p37 monoclonal antibody has an obvious effect on homotypic contact inhibition. The outward movement of the cells from the explants appeared to be unchanged.

It can be speculated that the permease which includes p37 changes the local concentration of its substrate or substrates. Such local changes may be adequate to influence the mechanisms of contact inhibition in the cellular host. P37-specific Fab would simply inhibit the permease activity. Fab may inhibit the invasivity of p37-free cells by binding to and partially masking a related cellular permease.

P37 may bind to a specific protein on the surface of mamalian cells. This 'receptor' would either be a component of the invasion machinery or part of the contact inhibition system. In the first case p37 could somehow stimulate the activity of the invasion machinery. Fab treatment would block this stimulation. In the latter case p37 would prevent the contact inhibition system to be active. Specific Fab may interfere in the blockade sterically. In p37-free cells the specific Fab treatment may mask and block a correlated cellular protein which normally interacts stimulatingly with the invasion machinery or specific Fab must interrupt the binding contact of a cellular p37 analog with the contact inhibition machinery.

Further Work

The present work raises a number of questions.

- (1) What is the function of p37 in mycoplasma cells? Although the DNA sequence data suggest the operon of which the p37 gene is a part may code for a permease complex, a computer search has found no known protein with significant homologies to p37. Modifying the activity of a functionally similar protein in tumor cells may modulate their invasive behavior.
- (2) Does p37 bind to a specific receptor on tumor cells? Is this interaction responsible for the effects of p37 on tumor cell invasion? The effects could eventually be narrowed down to a specific region of p37 and synthetic peptides used to modulate invasion *in vitro*.
- (3) What is the antigen or antigens to which the anti-p37 Fab binds on p37-free cells?
- (4) How does removal or addition of p37 (ie. Mycoplasma hyorhinitis) to cells affect their invasionary behavior *in vivo*? Do monoclonal antibodies directed against p37 reduce the invasiveness of tumor cells *in vivo*?

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ABBREVIATIONS

BSA	Bovine serum albumine
CM 52	Carboxymethyl cellulose
cpm	Counts per minute
DBA	3',3-diaminobenzidine tetrahydrochloride
DAPI	4,-6-diamidino-2-phenyl-indoldihydrochloride
DEAE DE 52	Dethylaminoethyl cellulose
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DTT	1,4-Dithio-DL-threitol
EDTA	Ethylene-diamine tetra acetate salt
Fab	Monovalent antibody
FCS	Fetal calf serum
IgG	Immunoglobulin G
HPLC	High performance liquid chromatography
kd	kilodalton
mAb	Monoclonal antibody
p37	Mycoplasma protein, 37 kilodalton
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphat buffered saline
SDS	Sodiumdodecyl sulfate

SUMMARY

The invasive behavior of a mouse fibrosarcoma cell line (FS9) can be inhibited *in vitro* by Fab fragments directed against a 37 kilo dalton protein (p37) associated with the surface of the tumor cells (Steinemann et al., 1984 a,b). We have used Abercrombie's confronted explant assay and time lapse film analysis to study the effects of p37 on the behavior of tumor cells *in vitro*.

1. A variety of cell lines were positive for p37. P37-negative cell lines also bound low levels of a p37-specific monoclonal antibody, indicating an additional protein associated with the cell surface shares an epitope with p37.
2. Incubation of p37-negative cells with the culture supernatant of FS9 cells resulted in the appearance of p37 after 5-10 days. This effect could be prevented if the anti-mycoplasma agent BM-cycline was included in the medium. A number of different mycoplasmas were identified in the cell lines used. P37 was exclusively associated with *Mycoplasma hyorhinis*.
3. The confronted explant assay was used to study the invasion of chicken heart fibroblasts by tumor cells. Tumor cells carrying p37 (i.e. infected with *Mycoplasma hyorhinis*) were highly invasive. Fab fragments directed against p37 strongly inhibited this invasion. The removal of p37 by BM-cycline treatment reduced the invasiveness of tumor cells, but the reduction was less than that observed when Fab was added to p37-positive cells. Addition of Fab to invasive, p37-negative cells also reduced their invasivity, once again suggesting tumor cell surface antigen is recognized by anti-p37 Fab. The invasive behavior of the non-invasive mouse L929 cells or chicken heart fibroblasts was not affected by the Fab. Non-specific Fab had no effect on the invasion of tumor cells.
4. The collisions between FS9 cells and chicken heart fibroblasts were filmed and analysed. FS9 cells carrying p37 (i.e. *Mycoplasma hyorhinis*) exhibited only weak contact inhibition of locomotion following collision with chicken heart fibroblasts. However, in the presence of p37-specific Fab strong contact inhibition was observed. P-37 negative FS9 cells were moderately contact inhibited and strong contact inhibition resulted from adding p37-specific Fab.

The results are consistent with p37 stimulating the invasive behavior of tumor cells *in vitro* and reducing the contact inhibition of locomotion. Blocking p37 with specific antibody can reverse these effects. Moreover, the antibody apparently recognizes an additional antigen(s) on tumor cells, thereby inhibiting invasiveness and causing cells to exhibit normal contact inhibition following heterotypic collision.

ZUSAMMENFASSUNG

Die Zellen der mausfibrosarcoma Zelllinie FS9 sind invasiv *in vitro*. Fab Fragmente gegen ein mit der Zelloberfläche assoziiertes 37 Kd Protein (p37) hemmen die Invasivität *in vitro* (Steinemann et al., 1984 a,b). Mit "Abercrombie's confronted explant test" und Zeitrafferfilmaufnahmen von Einzelzellkollisionen wurde die Wirkung von p37 auf Tumorzellen *in vitro* untersucht.

1. Verschiedenste Zelllinien waren p37-positiv. P37-negative Zellen absorbierten auch kleine Mengen von p37-spezifischen monoklonalen Antikörpern. Dies wies auf ein zusätzliches, zelloberflächenassoziiertes Protein hin, mit ähnlichem oder gleichem Epitop wie dasjenige in p37.

2. P37 erschien nach 5-10 Tagen in p37-negativen Zellen, wenn diese mit Mediumüberstand von FS9 Zellen inkubiert wurden. Dieser Effekt konnte unterbunden werden, wenn dem Medium ein Mycoplasmaantibiotika (BM-cycline) beigefügt wurde. Verschiedenste Arten von Mycoplasmen wurden von den verwendeten Zelllinien isoliert und identifiziert. P37 war ausschliesslich mit Mycoplasma hyorhinitis assoziiert.

3. Die *in vitro* Invasion von Tumorzellen in Hühnerherz Fibroblasten wurde mit "Abercrombie's confronted explant assay" untersucht. P37-positive Zellen (dh. infiziert mit Mycoplasma hyorhinitis) waren invasiv. Fab Fragmente gegen p37 hemmten diese Invasion stark. Das Verschwinden von p37 und Mycoplasmen nach einer Behandlung der Zellen mit BM-cycline war gekoppelt mit einer reduzierten Invasivität. Dieser Effekt war aber nicht so stark wie die Wirkung von Fab Fragmenten auf p37-positive Zellen. Das Beifügen von Fab Fragmenten zu p37-freien Zellen resultierte ebenfalls in einer verminderten Invasivität. Dies wies ein weiteres Mal auf das Vorhandensein eines Zelloberflächenantigens hin, welches von Fab Fragmenten erkannt wurde. Das Kontaktverhalten von nicht invasiven L929 Mauszellen und Hühnerherzfibroblasten wurde durch Fab Fragmente nicht beeinflusst, wie auch unspezifische Fab's keinen Effekt auf Tumorzellen zeigten.

4. Einzelzellkollisionen zwischen FS9 Zellen und Hühnerherzfibroblasten wurden gefilmt und analysiert. P37-positive FS9 Zellen zeigten eine sehr schwache Kontaktinhibition bei einer Kollision mit einem Hühnerfibroblasten. In Gegenwart von Fab Fragmenten waren diese FS9 Zellen sehr stark kontaktgehemmt. P37-negative FS9 Zellen waren leicht invasiv. Die Zugabe von p37-spezifischen Fab's bewirkte wiederum eine starke Kontakthemmung.

Die Uebereinstimmung der Resultate der Filmanalysen mit den "confronted explant assays" galt sowohl für den invasionsstimulierenden Effekt von p37 wie auch für die invasivitätsvermindernde Wirkung von Fab Fragmenten *in vitro*. Der Antikörper schien auf den Tumorzellen ein zusätzliches Antigen oder zusätzliche Antigene zu maskieren und damit eine normale Kontakthemmung bei heterotypischen Kollisionen auszulösen.

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